PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/82, 15/54, 9/10, 5/14, A01H 5/00 | A2

(11) International Publication Number: WO 97/15678

(43) International Publication Date: 1 May 1997 (01.05.97)

(21) International Application Number: PCT/US96/17351

(22) International Filing Date: 25 October 1996 (25.10.96)

(30) Priority Data:
08/549,016 27 October 1995 (27.10.95) US

(60) Parent Applications or Grants
(63) Related by Continuation
US 08/372,200 (CIP)
Filed on 12 January 1995 (12.01.95)
US 08/549,016 (CIP)
Filed on 27 October 1995 (27.10.95)

(71) Applicant *(for all designated States except US)*: CALGENE, INC. [US/US]; 1920 Fifth Street, Davis, CA 95616 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: SHEWMAKER, Christine, K. [US/US]; 1409 Spring Creek, Woodland, CA 95695 (US).

(74) Agents: RAE-VENTER, Barbara et al.; Rae-Venter Law Group, P.C., Suite 440, 260 Sheridan Avenue, Palo Alto, CA 94306 (US).

(81) Designated States: AU, CA, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: MODIFICATION OF SOLUBLE SOLIDS USING SUCROSE PHOSPHATE SYNTHASE ENCODING SEQUENCE

(57) Abstract

This invention relates to methods for the utilization of sucrose phosphate synthase encoding sequences to modify the soluble solids in plant sink tissue and to modified plants, plant tissues and plant parts. The method finds use for example for changing the sweetness of plant parts such as fruits and tubers.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

MODIFICATION OF SOLUBLE SOLIDS USING SUCROSE PHOSPHATE SYNTHASE ENCODING SEQUENCE

INTRODUCTION

Technical Field

The present invention is directed to compositions and methods related to modification of the sweetness of selected plant tissues. The invention is exemplified by plants, plant parts, and plant cells transformed with one or more copies of a transgene comprising DNA encoding SPS and a transcriptional initiation region functional in plants.

Background

Sucrose is one of the primary end products of photosynthesis in higher plants. It is also the major carbohydrate transported to sucrose accumulating, or carbon sink, tissues for plant growth and development. Plant regions, such as leaf tissue, where sucrose is synthesized are commonly referred to as sucrose source tissue. Plant storage organs, such as roots or tubers, and fruits are examples of sink tissues. The sucrose translocates from the mature leaf (source) to any tissue requiring photoassimilate (sink), especially growing tissues including young leaves, seeds, and roots. Difficulties in the purification of sucrose phosphate synthase (SPS) from plants have interfered with efforts to characterize this enzyme. SPS catalyses the formation of sucrose phosphate, the sucrose precursor molecule, from fructose-6 phosphate and UDP-glucose in photosynthetically active plant cells. Sucrose phosphatase then acts on the sucrose phosphate moiety, in an irreversible reaction, to remove the phosphate and to release sucrose.

SPS is considered a rate limiting enzyme in the pathway providing sucrose to growing tissue, therefore the study of SPS and its activity is of special interest. In a recent publication, Walker and Huber, *Plant Phys.* (1989) *89*:518-524, the purification and preliminary characterization of spinach *(Spinachia oleracea)* SPS was reported. However, monoclonal antibodies specific to the spinach SPS were found to be non-reactive with all other plants tested, "closely related" and "relatively unrelated species", including corn (*Zea maize*), soybean (*Glycine max*), barley (*Hordeum vulgare*), and sugar beet (*Beta vulgaris*). Thus, additional purified sources of SPS enzyme are needed for effective characterization of this factor. Especially of interest is the characterization of the corn SPS because of its very high export rates, as compared for example, to SPS levels of activity as found in the leaves of soybean.

With the advent of biotechnology, the ability to modify various properties of plants, especially agronomically important crops, is of interest. In this regard, it would be useful to determine the coding sequence for an SPS gene to probe other crop sources, to use such coding sequences to prepare DNA expression constructs capable of directing the expression of the SPS gene in a plant cell and to express a DNA sequence encoding an SPS enzyme in a plant to measure the effects on crop yield due to the increased rate of sucrose translocation to growing tissues.

Relevant Literature

The following references are related to expression of SPS in transgenic plants: Somnewald, *et al.* (1994) *Plant, Cell and Environment 17*:649-658; Worrell, *et al.* (1991) *The Plant Cell 3*:1121-1130; Micallef, *et al.* (1995) *Planta 196*:327-334; Foyer, *et al.* (1994) *Plant Physiol., 105*(S), 23; Galtier *et al.* (1993) *Plant Physiol. 101*:535-543; and PCT Application No. WO 94/00563. The following references are related to isolation of DNA encoding SPS: Valdez-Alarcon *et al.*, (1996) *Gene 170*(2):217-222; Sakamoto *et al.*, (1995) *Plant Science* (Shannon) *112*(2):207-217; Heese *et al.*, (1995) *Mol. Gen. Genet.*, *247*(4):515-520; Klein *et al.*, (1993) *Planta 190*(4):498-510; Salvucci *et al.*, (1993) *Plant Physiol.*, *102*(2):529-536; Sonnewald *et al.*, (1993) *189*(2):174-181; and Herrera-Estrella *et al.*, (1991) *J. Cell Biochem. Suppl.* 0 (15 Part A) 148. PCT Application WO 94/00563 discloses antisense potato SPS placed behind a tuber promoter and used to alter the sucrose levels in potato. Acid invertase encoding sequences are described by Klann *et al.*, (*Plant Phys.* (1992) *99*:351-353).

SUMMARY OF THE INVENTION

Methods for modifying the sweetness of plant sink tissues are provided in which sucrose phosphate synthase (SPS) activity and/or invertase activity in plant tissues are manipulated Also provided are nucleic acid constructs, vectors, plant cells, plant parts and plants containing at least one exogenously supplied copy of an SPS gene. The invention finds use in modifying carbohydrate partitioning in plant tissues and/or parts, which in turn can be used to alter plant growth, soluble solid content and/or sweetness, and/or to alter the sensitivity of plant growth to temperature and/or to levels of carbon dioxide and oxygen.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an SDS-PAGE profile of corn SPS at various stages of SPS purification and the quality of the final preparation. Using an 8.5% acrylamide gel.

reducing conditions and staining with silver nitrate. The abbreviations used are as follows: M: Standard of molecular weight B-Galactosidase (116 kd), bovine Albumin (68 kd), Egg Albumin (45 kd), carbonic anhydrase (29 kd); H: Heparin fraction, 30 micrograms of proteins per well; FP: Final Preparation, 7.5 micrograms of proteins per well; FE: Final Extract, 7.5 micrograms of proteins per well; D: Fast-Flow DEAE fraction, 78.5 micrograms of proteins per well.

Figure 2 shows the results of a Western analysis of SPS using monoclonal antibodies. In Fig. 2A, membrane is incubated in the presence of the SPB3-2-19 antibody; in Fig. 2B, membrane is incubated in the presence of an antibody not directed against SPS (negative control anti-neomycin monoclonal antibody); in Fig. 2C, membrane is incubated in the presence of the SPB13-2-2 antibody. The abbreviations used are as follows: M: standards of molecular weight radio-labeled by I-125, (NEX-188 NEN) B-Galactosidase (116 kd), bovine albumin (68 kd), carbonic anhydrous (29 kd), trypsin inhibitor (20.1 kd), Alpha-Lactalbumin (14.4 kd), 150,000 cpm per lane; PA: proteins obtained after immunoaffinity chromatography (see below) with the SPB13-2-2 monoclonal antibody, about 40 micrograms of proteins per lane; H: Heparin fraction, about 40 micrograms of protein per lane.

Figure 3 shows peptide sequences (SEQ ID NOS: 1-5) derived from SPS protein. All peptides are typed N→C terminal.

Figure 4 shows oligonucleotides used for the PCR reactions CD3 (SEQ ID NOS: 10-11) and CD4 (SEQ ID NOS: 12-13) in relation to the peptides (antisense sequences are presented upside down). Arrows point to the direction the oligonucleotides will prime the polymerase.

Figure 5 shows the characterization of CD3 and CD4 PCR reactions. Figure 5A shows agarose gel electrophoresis of CD3 and CD4 PCR reactions. The sizes are given in kb. Figure 5B shows autoradiograph of Southern blot of CD3 and CD4 PCF reactions probed with oligonucleotides 4k5 (SEQ ID NO: 14).

Figure 6 shows schematic diagrams representing SPS cDNA and selected clones. The upper bar represents the entire 3509 bp combined map. Translation stop and start paints are indicated.

Figure 7 shows the assembled SPS cDNA sequence (SEQ ID NO: 6). The sequences of clones SPS 90, SPS 61 and SPS 3 were fused at the points indicated in Fig. 2. The SPS reading frame is translated (SEQ ID NOS: 6-7). All SPS protein derived peptide sequences are indicated.

Figure 8 shows Western blots demonstrating the characteristics of rabbit SPS 90 and SPS 30 antisera. The abbreviations used are: pAS** = preimmune serum, SPS 30 rabbit ; AS** = immune serum anti-SPS 90. Molecular weight markers at left, where indicated.

S = SPS 120 kd polypeptide ; S* = SPS 90 kd polypeptide ; S** = SPS 30 kd polypeptide.

Figure 9 shows analysis of protein from a 30-day old corn plant. Figure 9A shows a Comassie Blue-stained gel of total protein isolated from a 30 day old corn plant. M = size marker ; R = roots ; 1-8 = leaf numbers counting from the bottom of the plant. Leaf 5 has been cut into 5 segments from the leaf tip (5a) to the end of the sheath (5c). PEP = phosphoenolpyruvate carboxylase. Figure 9B shows the results of Western blot analysis using a mixture of antiSPS 30 and antiSPS 90 antisera against total plant protein isolated from a 30 day old corn plant. The signal corresponding to SPS appears at 120-140 kd.

Figure 10 shows a schematic summary of a construction of plasmids pCGN627, pCGN639 and pCGN986. Figure 10A shows construction of pCGN627; Figure 10B shows construction of pCGN639; and Figure 10C shows construction of pCGN986.

Figure 11 shows partitioning between starch and sucrose as a function of temperature. The squares are data from control UC82B plants while triangles are data from transgenic tomatoes expressing SPS on a Rubisco small subunit promoter (pCGN3812).

Figure 12 shows maximum rates of photosynthesis for regenerated control (solid bars) and pCGN3812-24 transgenic (open bars) potatoes at three weeks after (panel A) and seven weeks after (panel B) planting.

Figure 13 shows tuber dry mass for regenerated control (solid bars) and pCGN3812-24 transgenic (open bars) potatoes add 35 and 70 Pa carbon dioxide in highlight growth chambers (Figure 13A) and open top chambers in the field (Figure 13B).

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods for modifying the solids content of plant sink tissue which use a construct encoding SPS for example as a way of increasing the sweetness of fruit. The soluble solids include simple sugars, but also can include certain soluble polymers, and other soluble cell components. Total solids include more complex carbon compounds, such as starches and cellulose. The method provides for increasing the total solids in a plant sink tissue so that total solids are modified from a given ratio of total solids per unit weight of sink tissue, as measured in control plant cells, to a different ratio of total solids per unit weight of sink tissue. The amount of sucrose available to growing tissues in the plant is increased, and the increased sucrose results in increased total solids per unit weight in the sink tissues of the plant.

The method generally comprises growing a plant having integrated into its genome a construct comprising as operably linked components in the 5' to 3' direction of transcription, a transcription initiation region functional in a plant cell and a DNA encoding SPS. The transcription initiation region may be constitutive or tissue specific. By tissue

specific is intended that the region is preferentially expressed in cells of a particular plant tissue or part, for instance fruit or leaf as compared to other plant tissues. In one embodiment the method produces sink tissue having increased carbon as soluble solids, as an increased ratio of soluble solids per unit weight of sink tissue, as compared to that measured in control plant cells. This results from the increased levels of sucrose generating an increased rate of transportation of the available sucrose into the carbon sink tissue. In another embodiment, a method is provided to modify the soluble solids ratios in sink tissue, such as the ratio of sucrose to fructose, as compared to that measured in control plant cells or tissue. If the increased soluble solids in said sink tissue comprises fructose, a phenotype will result having an increased sweetness as opposed to the control tissue. A method is also disclosed, however, whereby a decreased ratio of fructose to sucrose, and whereby a reduced sweetness phenotype may be produced.

The use of constructs comprising encoding sequences to other sucrose metabolizing enzymes, such as acid invertase, or the utilization of such enzymes which are endogenous to the plant sink cells, can be advantageously used with this invention. For instance, acid invertase can be expressed in the cells or sink tissue from an expression construct, or, alternatively, the sink tissue can be prevented from converting sucrose to fructose and glucose by the use of an antisense acid invertase construct, whereby cells of the sink tissue will have a decreased acid invertase activity, and thereby a decreased ratio of fructose to sucrose as compared to cells in a control sink tissue. Fruit having increased total soluble solids and/or modified or increased fructose levels, as measured per unit weight are provided and include fruits such as tomato, strawberry and melon. The fruit has a modified sweetness phenotype, either from a total increase in sweetness by percentage of fruit weight, or from an increased ratio of fructose to sucrose in the soluble solids in the fruit.

Transgenic plants and plant parts are provided which have altered carbon partitioning and end-product synthesis through expression of a transgene required for sucrose synthesis. The transgenic plants, cells and plant parts such as leaf, fruit and root are characterized by modified levels of SPS activity compared to controls. By "modified SPS activity" is intended an increase or decrease in sucrose synthesis. Modification of SPS activity according to the subject invention alters the carbon partitioning between source tissue and sink tissue through an increase or decrease in sucrose synthesis. Altered carbon partitioning is manifested by one or more changes in development, growth and yield through modification of end-product synthesis and conversion in general. The protein and DNA encoding SPS of the subject invention is obtainable from any number of sources which contain an endogenous SPS. Among the preferred SPSs are those obtainable from corn or derived from corn SPS protein or nucleic acid using antibody and/or nucleic acid probes for SPS identification, amplification and isolation. The subject invention also

provides a variety of SPS transgenes which have different promoter regions to regulate the transcription and level of SPS activity in plants or plant parts in a tissue-specific and growth-dependent manner. Among the preferred promoter regions are those which provide for leaf, fruit and/or root specific expression of SPS. Preferably, the DNA encoding a SPS of interest in operably linked in a sense or antisense orientation to a selected transcription initiation region to provide for a sufficient level of expression of SPS in the desired tissue or tissues.

An advantage of increasing or decreasing SPS activity is the modification of sucrose synthesis, which is a key metabolic product that affects the interface between end-product synthesis and carbon partitioning for most plant systems. By "end-product" synthesis is intended the metabolic product interface between photosynthesis and plant growth and development. Information flow across the interface may occur by mass action or by signal transmission and transduction. Mass action effects occur when an increase in photosynthesis leads to faster growth resulting from an increase in the availability of photosynthate. Conversely, mass action feedback occurs when accumulation of end-products reduce the rates of photosynthetic reactions. Thus, an advantage of the subject invention is that modification of sucrose synthesis through SPS activity provides a central control point for modifying carbohydrate partitioning through end-product synthesis in a source tissue such as leaf and end-product conversion in a sink tissue such as growing leaf, fruit or root. For example, modulation of photosynthetic metabolism through expression of exogenous SPS is advantageously used to alter the synthesis of end-products such as starch, sucrose glucose, fructose, sugar alcohols, and glycine and serine from photorespiratory metabolism. SPS preferably is used to modulate end product synthesis of non-phosphorylated products of metabolism.

Another advantage of the subject invention is that altering SPS activity provides a means for altering plant growth and yield of specific plant cells, plant tissues, plant parts and plants. In addition, by modulating the ability of a plant to synthesize sucrose, the growth response of a plant under a variety of different environmental conditions can be affected including carbon dioxide utilization, oxygen sensitivity, temperature-dependent growth responsiveness and expression of endogenous genes responsive to sugar content in general. Manipulation of growth conditions also permits the modulation of metabolism and the activity of the SPS transgene, for example, through light-mediated activation or deactivation of the SPS transgene and its product. Another advantage is the modification of overall soluble solids such as starch, sucrose, glucose and fructose in sink tissue such as fruit or root. SPS activity and sugar content also permit manipulation of endogenous gene expression and/or enzyme activity in the plant, such as the endogenous acid invertase found in ripening fruit to increase glucose and fructose levels as well as acid content. An additional advantage is that the onset of flowering, fruit number, mass, dimensions, and

overall morphology can be modified by altering carbon partitioning. Thus, the subject invention permits the obtention of any transgenic plant or plant part which have any one of several readily selectable phenotypes related to SPS transgene expression and SPS protein activity.

In the subject invention, purification of corn SPS protein is exemplified. By "protein" is intended any amino acid sequence, including a protein, polypeptide, or peptide fragment, whether obtained from plant or synthetic sources, which demonstrates the ability to catalyze the formation of sucrose phosphate. An SPS of this invention includes sequences which are modified, such as sequences which have been mutated, truncated, increased in size, contain codon substitutions as a result of the degeneracy of the DNA code, and the like as well as sequences which are partially or wholly artificially synthesized, so long as the synthetic sequences retain the characteristic SPS activity. SPS from sources in addition to corn are obtainable by a variety of standard protocols employing protein properties, amino acid and nucleic acid information derived from corn SPS. For example, antibody or nucleic acid probes derived from sequencing information permit isolation of a gene or parts of the gene including genomic DNA and cDNA encoding the target SPS of interest. For this purpose, degenerate and non-degenerate probes from hybridization studies with parts or all of the corn SPS sequence can be used for identification, isolation and amplification of a gene or fragments encoding the SPS of interest. The SPS gene or fragments are assembled and evaluated by conventional recombinant DNA and biochemical techniques, and through nucleic acid and amino acid sequence database comparisons. As an example, SPSs derivable from corn SPS sequences in this manner include potato, spinach, rice and sugar beet. *In vitro* and *in vivo* expression systems can be used to produce and test the SPS. The SPS activity can be evaluated by measuring formation of sucrose phosphate from fructose-6-phosphate and UDP-glucose substrates.

In order to obtain the nucleic acid sequences encoding the SPS, especially corn SPS, substantially purified SPS was required. As demonstrated more fully in the examples, corn SPS purified 500-fold was obtained in small quantities which were then ultimately used to obtain the peptide sequence which in turn led to the determination of the cDNA sequence.

Among the preferred proteins of the invention are the proteins having the above definition with a molecular weight from about 110 to about 130 kd, having the form of a monomer, a dimer or a tetramer and their derivatives, comprising at least one peptide having the following amino acid sequence:

Thr-Trp-Ile-Lys (SEQ ID NO: 1)

Tyr-Val-Val-Glu-Leu-Ala-Arg (SEQ ID NO: 2)

Ser-Met-Pro-Pro-Ile-Trp-Ala-Glu-Val-Met-Arg (SEQ ID NO: 3)

Leu-Arg-Pro-Asp-Gln-Asp-Tyr-Leu-Met-His-Ile-Ser-His-Arg (SEQ ID NO: 4)

Trp-Ser-His-Asp-Gly-Ala-Arg (SEQ ID NO: 5)

The invention also relates to a process to prepare proteins as above defined, having the following steps: (a) extracting SPS from parts containing SPS, which are preserved at low temperature, by grinding, centrifugation and filtration; (b) increasing the rate of SPS extraction from the extract so obtained by precipitation in an appropriate solvent, centrifugation and solubilization of the precipitate in a buffer solution; (c) purifying the protein so obtained by chromatography and, if desired, (d) preparing hybridomas, and monoclonal antibodies from an antigenic solution obtained at step (a), (b), or (c) above; (e) screening the hybridomas and raising monoclonal antibodies specifically directed against SPS; and (f) further purifying the SPS obtained at step (a), (b), or (c) with the monoclonal antibodies prepared.

The invention more precisely relates to a process of preparation of corn SPS having the following steps: (a) extracting SPS from parts of corn plants by grinding, centrifugation, and filtration; (b) increasing the rate of SPS extraction from the extract so obtained by precipitation in polyethyleneglycol (PEG), centrifugation and solubilization of the precipitate obtained in a buffer solution; (c) purifying the protein so obtained by low pressure anion exchange chromatography and by chromatography on heparin sepharose, then by anion exchange high performance chromatography; (d) purifying the active pools by passage on two high performance chromatography columns, and if desired; (e) preparing hybridomas and monoclonal antibodies from an antigenic solution prepared from steps (a), (b), or (c); (f) screening the hybridomas and raising the monoclonal antibodies specifically directed against SPS; and (g) purifying the SPS preparation with the monoclonal antibodies so obtained.

Preferably the corn is a corn Pioneer corn hybrid strain 3184, the parts of plants are leaves which are kept at low temperature, for example between -50°C and -90°C, and purification in the polyethyleneglycol is realized first by precipitating at a final concentration in PEG about 6%, and then by precipitating at a final concentration of about 12%. The various chromatographies are performed in the following way: 1st chromatography, DEAE sepharose; 2nd chromatography, heparin sepharose (at this stage, the preparation obtained may be kept several days without loss of activity); 3rd chromatography, Mono Q chromatography; 4th chromatography, HPLC hydroxyapatite; and 5th chromatography, HPLC hydroxyapatite.

A variety of additional protein fractionation methods can be combined to generate a suitable purification scheme for SPS proteins and peptides from corn and those in addition

to corn. If only very small amounts of denatured protein are needed, a high resolution technique may be used such as two-dimensional gel electrophoresis to obtain the protein in one step. When retention of activity is desired, a series of purification steps are designed to take advantage of different properties of the SPS of interest such as precipitation properties, charge, size, adsorptive properties and affinity properties as demonstrated for corn SPS.

In general, purification follows the initial extraction and preparation of total protein, bulk precipitation followed by chromatographic procedures such as ion exchange, adsorption, gel filtration, affinity resins and non-denaturing electrophoresis methods so as to be substantially free from other proteins, particularly proteins of the source tissue. By "substantially free from other proteins" is meant that the protein has been partially purified away from proteins found in the source tissue or organism. Such a protein of this invention will demonstrate a specific enzymatic activity of at least greater than 0.05, more preferably at least greater than at least 0.30, wherein specific enzymatic activity (sA) is measured in units which correspond to 1 µmole (micromole) of sucrose formed per minute per mg of protein at 37°C. In a more preferred embodiment, the protein will demonstrate even more improved sA and increased purification factors (see, Table 5).The proteins can be further purified if desired, when retention of activity is less important, by electrophoretic procedures including native or denaturing polyacrylamide gel electrophoresis, isoelectric focusing and two dimensional gel electrophoresis. During the different steps of purification and thereafter, the SPS activity can be measured by two methods: (a) a method based on a colorimetric test or resorcinol test; and (b) a method based on the amount of one of the products formed during the transformation reactions where SPS is involved. Both methods are detailed in the experimental part detailed hereunder. The exemplified invention relates to the enzyme comprising a corn SPS having a molecular weight from about 110 to 130 kilodalton (kd) and a specific activity of greater than 0.05 U. The invention relates more particularly to the enzyme comprising a corn SPS having a specific activity of about 25 U. Antibodies to SPS are prepared as follows, or by other methods known to those skilled in the art. Mice are immunized with several injections of enzymatic preparations. Different kinds of mice may be used, for example BALB/c. The antigen can be provided in complete Freunds adjuvant then in incomplete Freunds adjuvant. Several injections in mice are realized: good results have been obtained with three injections of Mono Q, pools, (see above purification scheme) followed by three injections of final pools (days 0, 14, 27, 60, 90 and 105 for example). The first injections are administered sub-cutaneously, for example in the cushions, and the feet, the last injection is administered intravenously, in the tail for example. The preparation of spleen cellular suspensions from animals immunized as described above is made in a conventional

way. The steps of fusion with myeloma cells, of conservation of the hybridoma, of cloning, of antibodies production are made by conventional ways. To detect the hybridoma secreting the monoclonal antibodies raised against the antigen, two methods are used to select antibodies: a method of detection of antibodies as inhibitor of SPS activity; and a method of detection of antibodies precipitating SPS activities. In a preferred embodiment, these methods are the methods described in the experimental section detailed hereunder.

Among the objects of the invention, are also provided lines of hybridoma cells, and in particular hybridoma cells described as: SPA 2-2-3 : I-971; SPA 2-2-22 : I-970; SPA 2-2-25 : I-972; SPB 3-2-19 : I-973; SPB 5-2-10 : I-974; SPB 5-4-2 : I-975; SPB 13-1-7 : I-976; and SPB 13-2-2 : I-977. Deposits of these hybridoma cells were made at the C.N.C.M. (Institut Pasteur Paris) on June 11, 1990. The invention relates also to monoclonal antibodies specifically directed against SPS.

The invention relates also to a process of preparation of proteins as defined above characterized in that a preparation containing the so-called proteins is purified on a chromatography column having monoclonal antibodies as defined above specifically raised against the proteins.

The invention relates also to cDNA coding for proteins as defined above, especially cDNA coding for corn SPS. Among the preferred cDNA, most preferred is cDNA comprising a nucleotide sequence represented in Figure 7 (SEQ ID NO: 6). Thus, this invention relates to an extrachromosomal DNA sequence encoding a SPS as defined above. Any DNA sequence which is not incorporated into the genome of a plant is considered extrachromosomal, i.e., outside of the chromosome, for purposes of this invention. This includes, but is not limited to cDNA, genomic DNA, truncated sequences, single stranded and double stranded DNA. In a preferred embodiment, the DNA sequence is cDNA. In a different preferred embodiment, the DNA sequence is obtainable from corn or is derived from the corn DNA sequence.

Among the preferred proteins and nucleic acid sequences of the invention is corn SPS. The corn SPS is represented in Figure 1, which shows the presence of proteins at about 120, 95 and 30 kd. The proteins shown at 95 and 30 kd are considered to be breakdown products of the protein shown at 120 kd. The complete protein is believed to be a di- or tetrameric protein having as the basic sub-unit from about a 110 to about a 130 kd protein. The complete cDNA sequence of the corn SPS is shown in Figure 7 (SEQ ID NO: 6).

The cDNA coding for sucrose phosphate synthase has been prepared in the following way: (1) sequencing of peptide fragments from purified SPS. With the purified preparations of SPS previously obtained, following separation on an acrylamide gel, a 120 kd minor band (corresponding to the total protein sequence) and two 90 kd and 30 kd major bands are obtained. Both major polypeptides are separated by electrophoresis and

electroeluted. By trypsin digestion and sequencing of the fragments so obtained, the sequence of 5 peptides has been determined. This amino acid sequence makes it possible to determine the corresponding degenerate nucleotide sequence.

(2) Corn leaf isolation. Total RNA is isolated according to Turpen and Griffith (1986, *Biotechniques 4*:11-15) for poly(A) RNA preparation, the standard oligo dT cellulose column is used.

(3) cDNA library construction. cDNA is synthesized using the protocol of a kit supplied by Promega except that M-MLV reverse transcriptase is used instead of AMV reverse transcriptase. The length of cDNA obtained is from 500 to several thousand base pairs. *Eco*RI linkers are added to the blunt ended cDNA and this material is cloned into a second generation lambda GT11 expression vector. Total library size is about $1.5x10^6$ plaques.

(4) Utilization of PCR to synthesizing a nucleotide sequence specific for SPS. The oligonucleotides derived from peptides B11 (SPS 30 kd) (SEQ ID NO: 3) and 4K (90 kd) (SEQ ID NO: 4) described in figure 3 are used as primers in a PCR reaction. It has been assumed that peptides derived from SPS 30 and SPS 90 are degradation products of protein SPS 120 kd, and that the peptides derived from SPS and SPS 90 are encoded by the same RNA.

With this hypothesis, by using in proper polarity pairs of oligonucleotides corresponding to the peptidic sequences in a PCR reaction, one may obtain the synthesis of the DNA, connecting the two location. Since it is a priori not know in which order the peptides are located relative to each other, one has to do the two different possibilities (Fig. 4). Only the oligonucleotide couple CD3 synthesizes a cDNA of defined length (1200 bp) (Fig. 5).

(5) cDNA library screening. When 250,000 lambda clones GT11 are screened using the 1200 bp long PCR cDNA, 16 positives are obtained. Sizes of the inserts ranged from 0.3 kb to 2.8 kb (see Fig. 6 for the two longest clones). The sequence is not complete in 5'. In a second round of library screening with a 400 bp DNA fragment corresponding to the most 5' fragment of the clone SPS 3, a SPS 61 clone extending further 5' without having the 5' end of the reading frame is obtained (Fig. 6).

(6) Creation and screening of a second cDNA library in order to clone the 5' sequence of cDNA coding for SPS. A oligonucleotide complementary to the 5' sequence of clone SPS 61 is used as a primer for cDNA synthesis. After second strand reaction is completed, the cDNA is cloned into bacteriophage lambda GT11. The library includes about one million clones. The SPS 90 and SP 77 were obtained by screening this library with SPS 61 (Fig. 6).

(7) The assembled SPS reading frame. DNA sequences which encode the SPS may

be employed as a gene of interest in a DNA construct or as probes in accordance with this invention. When provided in a host cell, the sequence can be expressed as a source of SPS. More preferred is the SPS sequence in a vegetal cell under the regulatory control of a transcriptional and translational initiation region functional in plants. Vegetal cell means any plant cell being able to form undifferentiated tissues as callus or differentiated tissues as embryos, parts of plants, whole plants or seeds. Plants means for example plants producing grain seeds such as cereals, and includes wheat, barley, corn, and oat; leguminous plants such as soybean; oleaginous plants such as turnesol; tuberous plants such as potato; plants with roots such as beet; and fruit such as tomato. The sucrose phosphate synthase is a key enzyme, in sucrose regulation mechanisms, but also in carbon partitioning regulation between starch and sucrose during photosynthesis (see J. Preiss, *Tibs* January 1984, page 24, or Stitt and Coll, (1987) *Biochemistry of Plants, 10*:3-27). Of particular interest are plants of the nightshade family Solanaceae, including the genetically similar but physiologically disparate plants potato (*Solanium tuberosum*) and tomato (*Hycopersicon esculentum*).

When provided in a DNA construct for integration into a plant genome, the sequence can encode a sense strand or an anti-sense strand. By increasing the amount of SPS available to the photosynthetically active plant cell by the expression of additional SPS, an increased flow of sucrose can be provided to growing tissues resulting, for example, in increased plant yields; by decreasing the amount of SPS available to the photosynthetically active plant cell, the rate of sucrose release from the plant cell may be hindered, resulting in less new plant growth. Controlling the rate of transport and the amount of sucrose available to growing tissues can be used to increase or decrease the total solids in a plant sink tissue from a given ratio of total solids per unit weight sink tissue. Total solids include soluble solids and insoluble solids such as sugars, starches and cellulose. Of particular interest are the soluble solids, which include the sugars sucrose, fructose, and glucose, soluble organics, polymers and other soluble components of cells. Increased total solids in a plant sink tissue may be in the form of an increase in glucose and/or fructose levels. Where the increase comprises fructose, for example, the resulting phenotype is increased sweetness. Where fructose levels are lowered a reduced sweetness phenotype is produced. Of particular interest is fruit having a modified sweetness phenotype. Increasing or decreasing the flow and/or amount of sucrose available to fruit tissue increases or decreases the conversion of sucrose to glucose and fructose by acid invertase, and thus the sweetness of fruit. In tomato fruit, for example, glucose and fructose are produced from sucrose by a vacuolar acid invertase that is active during fruit ripening. As fructose is twice as sweet on a molar basis as glucose, an increase in fructose levels or a fructose to glucose ratio can result in an increased sweetness of the fruit. Of

particular interest is fruit of the plant family Solanaceae. Sink tissue solids can be modified with SPS levels and/or activity in conjunction with endogenous sucrose and starch metabolizing enzymes, such as acid invertase for sucrose and glycogen synthase for starch. Modification can be used to enhance or inhibit enzymatic activity, for example through sense or antisense expression. By increasing or decreasing SPS activity in plants, the interaction between photosynthesis and the synthesis of end products, such as sucrose and starch, can be modified. Of particular interest is the modification of the starch to sucrose ratio in a vegetal cell through the expression of a transgene encoding SPS. Modifying the starch to sucrose ratio in vegetal cell may transduce the affect through end-product synthesis, signal transduction and/or translocation to other vegetal cells, particularly the vegetal cells of leaf, fruit and root. In some plants, the change in carbohydrate partitioning can also affect the sensitivity of the altered plant to carbon dioxide and oxygen. Increasing sucrose synthesis can result in greater capacity for photosynthesis at elevated carbon dioxide, particularly in the potato. Conversely, decreasing sucrose synthesis (increasing starch synthesis) induces oxygen insensitivity. Such an effect can be obtained by expressing antisense SPS.

A sucrose metabolizing enzyme can also be modified through sense or antisense expression. Sequences to be transcribed are ligated to the 3' end the plant transcription initiation region. In the sense constructs, an mRNA strand is produced which encodes the desired sucrose metabolizing enzyme, while in antisense constructs, an RNA sequence complementary to an enzyme coding sequence is produced. The sense strand is desirable when one wishes to increase the production of a sucrose metabolizing enzyme in plant cells, whereas the antisense strand may be useful to inhibit production of a related plant sucrose metabolizing enzyme. The inhibition of acid invertase in tomato fruit, for instance, can lead to fruit having elevated levels of sucrose in the tomato fruit. The sequence to acid invertase is known (Klann *et al.*, (1992) *Plant. Phys.* (1992) 99:351-353). Expression of other sucrose metabolizing enzymes may result in alterations to other carbon components, for instance the expression of starch synthesizing enzymes to act in concert with the increase availability of sucrose may result in increased starch levels in the sink tissue. The transformation of plants using glycogen synthesis enzymes (glgA, glgB and glgC) to modify starch compositions is described in U. S. Patent No. 5,349,123.

The presence of sucrose metabolizing enzyme sequences in the genome of a plant host cell may be confirmed, for example by a Southern analysis of DNA or a Northern analysis of RNA sequences or by PCR methods. In addition to sequences providing for transcriptional initiation in a plant cell, also of interest are sequences which provide for transcriptional and translational initiation of a desired sequence encoding a sucrose metabolizing enzyme. Translational initiation regions may be provided from the source of the transcriptional initiation region or from the gene of interest. In this matter, expression

of the sucrose metabolizing enzyme in a plant cell is provided. The presence of the sucrose metabolizing enzyme in the plant host cell may be confirmed by a variety of methods including an immunological analysis of the protein (e.g. Western or ELIZA), as a result of phenotypic changes observed in the cell, such as altered soluble solids content or by assay for increased enzyme activity, and the like.

Other sequences may be included in the nucleic acid construct providing for expression of the sucrose metabolizing enzymes ("expression constructs") of this invention, including endogenous plant transcription termination regions which will be located 3' to the desired sucrose metabolizing enzyme encoding sequence. For instance, transcription termination sequences derived from a patatin gene may be utilized when the sink tissue is potato tubers. Transcription termination regions may also be derived from genes other than those used to regulate the transcription in the nucleic acid constructs of this invention. Transcription termination regions may be derived from a variety of different gene sequences, including the *Agrobacterium*, viral and plant genes discussed above for their desirable 5' regulatory sequences. Further constructs are considered which provide for transcription and/or expression of more than one sucrose metabolizing enzyme. For example, one may wish to provide enzymes to plant cells of the sink tissue which provide for modification of the type of soluble solids to be produced therein, as well as for enhancing or otherwise modifying the increase or decrease in overall soluble solids production. An example of enzymes which may prove useful in modifying soluble solids ratios is the acid invertase enzyme.

In developing the nucleic acid constructs of this invention, the various components of the construct or fragments thereof will normally be inserted into a convenient cloning vector, e.g. a plasmid, which is capable of replication in a bacterial host, e.g. *E. coli*. Numerous vectors exist that have been described in the literature, many of which are commercially available. After each cloning, the cloning vector with the desired insert may be isolated and subjected to further manipulation, such as restriction, insertion of new fragments or nucleotides, ligation, deletion, mutation, resection, etc. so as to tailor the components of the desired sequence. Once the construct has been completed, it may then be transferred to an appropriate vector for further manipulation in accordance with the manner of transformation of the host cell.

The constructs of this invention providing for transcription and/or expression of sucrose metabolizing enzyme sequences of this invention may be utilized as vectors for plant cell transformation. The manner in which nucleic acid sequences are introduced into the plant host cell is not critical to this invention. Direct DNA transfer techniques, such as electroporation, microinjection or DNA bombardment may be useful. To aid in identification of transformed plant cells, the constructs of this invention may be further

manipulated to include plant selectable markers. The use of plant selectable markers is preferred in this invention as the amount of experimentation required to detect plant cells is greatly reduced when a selectable marker is expressed. Useful selectable markers include enzymes which provide for resistance to an antibiotic such as gentamycin, hygromycin, kanamycin, and the like. Similarly, enzymes providing for production of a compound identifiable by color change, such as GUS, or luminescence, such as luciferase are useful.

An alternative method of plant cell transformation employs plant vectors which contain additional sequences which provide for transfer of the desired sucrose metabolizing enzyme sequences to a plant host cell and stable integration of these sequences into the genome of the desired plant host. Selectable markers may also be useful in these nucleic acid constructs to provide for differentiation of plant cells containing the desired sequences from those which have only the native genetic material. Sequences useful in providing for transfer of nucleic acid sequences to host plant cells may be derived from plant pathogenic bacteria, such as *Agrobacterium* or *Rhizogenes*, plant pathogenic viruses, or plant transposable elements.

A sucrose metabolizing enzyme considered in this invention includes any sequence of amino acids, such as protein, polypeptide, or peptide fragment, which demonstrates the ability to catalyze a reaction involved in the synthesis or degradation of sucrose or a precursor of sucrose. These can be endogenous plant sequences, by which is meant any sequence which can be naturally found in a plant cell, including native (indigenous) plant sequences as well as sequences from plant viruses or plant pathogenic bacteria, such as *Agrobacterium* or *Rhizobium* species that are naturally found and functional in plant cells. It will be recognized by one of ordinary skill in the art that sucrose metabolizing enzyme sequences may also be modified using standard techniques of site specific mutation or PCR, or modification of the sequence may be accomplished in producing a synthetic nucleic acid sequence and will still be considered a sucrose biosynthesis enzyme nucleic acid sequence of this invention. For example, wobble positions in codons may be changed such that the nucleic acid sequence encodes the same amino acid sequence, or alternatively, codons can be altered such that conservative amino acid substitutions result. In either case, the peptide or protein maintains the desired enzymatic activity and is thus considered part of the instant invention. A nucleic acid sequence to a sucrose metabolizing enzyme may be a DNA or RNA sequence, derived from genomic DNA, cDNA, mRNA, or may be synthesized in whole or in part. The structural gene sequences may be cloned, for example, by isolating genomic DNA from an appropriate source, and amplifying and cloning the sequence of interest using a polymerase chain reaction (PCR). Alternatively, the gene sequences may be synthesized, either completely or in part, especially where it is desirable to provide plant-preferred sequences. Thus, all or a portion of the desired

structural gene may be synthesized using codons preferred by a selected plant host. Plant-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a particular plant host species. Other modifications of the gene sequences may result in mutants having slightly altered activity. Once obtained, a sucrose metabolizing enzyme may be utilized with the SPS sequence in a variety of ways.

Other endogenous plant sequences may be useful in nucleic acid constructs of this invention, for example to provide for transcription of the sucrose metabolizing enzyme sequences. Transcriptional regulatory regions are located immediately 5' to the DNA sequences of the gene of interest, and may be obtained from sequences available in the literature, or identified and characterized by isolating genes having a desirable transcription pattern in plants, and studying the 5' nucleic acid sequences. Numerous transcription initiation regions which provide for a variety of constitutive or regulatable, e.g. inducible, expression in a plant cell are known. Among sequences known to be useful in providing for constitutive gene expression are regulatory regions associated with *Agrobacterium* genes, such as for nopaline synthase (*Nos*), mannopine synthase (*Mas*), or octopine synthase (*Ocs*), as well as regions coding for expression of viral genes, such as the 35S and 19S regions of cauliflower mosaic virus (CaMV). The term constitutive as used herein does not necessarily indicate that a gene is expressed at the same level in all cell types, but that the gene is expressed in a wide range of cell types, although some variation in abundance is often detectable.

In providing for transcription and/or expression of the sucrose metabolizing enzyme sequences, for various reasons one may wish to limit the expression of these enzymes to plant cells which function as carbon sinks. Towards this end, one can identify useful transcriptional initiation regions that provide for expression preferentially in specific tissue types, such as roots, tubers, seeds or fruit. These sequences may be identified from cDNA libraries using differential screening techniques, for example, or may be derived from sequences known in the literature.

Many tissue specific promoter regions are known, such as the Rubisco small subunit promoter which preferentially is expressed in leaf tissue, the patatin promoter which is preferentially in potato tubers. Other transcriptional initiation regions which preferentially provide for transcription in certain tissues or under certain growth conditions, include those from napin, seed or leaf ACP, zein, and the like. Fruit specific promoters are also known, one such promoter is the E8 promoter, described in Deikman *et al.* (1988) *EMBO J.* 2:3315-3320; and DellaPenna *et al.* (1989) *Plant Cell 1*:53-63, the teachings of which are incorporated herein by reference. An E8-SPS construct (fruit-specific promoter) will express SPS in a fruit-specific manner, whereby the levels of sucrose produced in the fruit may be elevated. If coupled with antisense acid invertase, the increase in sucrose would be

maintained. This is a particular issue in tomatoes where acid invertase present in the fruit drives the production of glucose and fructose from sucrose..

The protein and DNA encoding SPS of the subject invention is obtainable from any source containing an endogenous SPS and can be wholly or partially synthetic. Among the preferred SPSs are those obtainable from corn. By "obtainable from corn" is meant that the sequence, whether an amino acid sequence or nucleic acid-sequence, is related to a corn SPS, including a SPS recovered through use of nucleic acid probes, antibody preparations, sequence comparisons or derivatives obtained through protein modeling or mutagenesis for example. Thus, one skilled in the art will readily recognize that antibodies, nucleic acid probes (DNA and RNA) and the like can be prepared and used to screen other plant sources for SPS and recover it. Typically, a homologously related nucleic acid sequence will show at least about 60% homology, and more preferably at least about 70% homology between the corn SPS and the given plant SPS of interest, excluding any deletions which may be present. Homology is found when there is an identity of base pairs and can be determined upon comparison of sequence information, nucleic acid or amino acid, or through hybridization reactions conducted under relatively stringent conditions, e.g., under conditions where there is a fairly low percentage of non-specific binding with corn SPS probes.

Probes can be considerably shorter than the entire sequence, but should be at least about 10, preferably at least about 15, more preferably at least 20 nucleotides in length. Longer oligonucleotides are also useful, up to full length of the gene encoding the polypeptide of interest. Both DNA and RNA probes can be used. A genomic library prepared from the plant source of interest can be probed with conserved sequences from corn SPS to identify homologously related sequences. Use of the entire corn SPS cDNA may be employed if shorter probe sequences are not identified. Positive clones are then analyzed by restriction enzyme digestion and/or sequencing. In this general manner, one or more sequences can be identified providing both the coding region, and the transcriptional regulatory elements of the SPS gene from such plant source. As an example, probes derived from corn SPS are used for isolating SPS from corn and sources in addition to corn. A probe or a battery of probes representing all or segments of the SPS coding region of corn SPS are preferably used. The corn SPS sequences can be compared by conventional gene bank searches and the conserved and nonconserved regions used in the design of additional probes if needed. In addition, the conserved and nonconserved regions for probe design are identifiable through standard hybridization techniques or, for example, by comparing amino acid and/or nucleic acid sequences of corn SPS to SPS sequences from diverse sources including rice, potato, sugar beet, spinach ,or *Arabidopsis thaliana*. which is a flowering plant member of the mustard family Brasicaceae.

In use, probes are typically labeled in a detectable manner (for example with ^{32}P-labelled or biotinylated nucleotides) and are incubated with single-stranded DNA or RNA from the plant source in which the gene is sought, although unlabeled oligonucleotides are also useful. Hybridization is detected by means of the label after single-stranded and double-stranded (hybridized) DNA or DNA/RNA have been separated, typically using nitrocellulose paper or nylon membranes. Hybridization techniques suitable for use with oligonucleotides are well known to those skilled in the art.

From the cDNA sequences, one skilled in the art can obtain the corresponding genomic DNA sequences related thereto to obtain the coding region of the SPS, including intron sequences, transcription, translation initiation regions and/or transcript termination regions of the respective SPS gene. The regulatory regions can be used with or without the SPS gene in various probes and/or constructs. The complete SPS reading frame can be assembled using restriction enzyme fragments of SPS 90, SPS 61 and SPS 3, see Fig. 6.

When expressed in *E. coli*, the SPS cDNA produces a protein which is recognized by anti-SPS antisera and has the same electrophoretic mobility as SPS extracted from corn leaves. We show that this *E. coli* SPS is as active as plant SPS, i.e. for complete enzymatic activity in *E. coli* no other plant factor is needed but the SPS cDNA.

Plants obtained by the method of transformation and containing fusions of SPS cDNA to tissue specific promoters in order to modify or alter the composition of certain plant organs are also included.

A DNA construct of this invention can include transcriptional and translational initiation regulatory regions homologous or heterologous to the plant host. Of particular interest are transcriptional initiation regions from genes which are present in the plant host species, for example, the tobacco ribulose biphosphate carboxylase small subunit (SSU) transcriptional initiation region; the cauliflower mosaic virus (CaMV) 35S transcriptional initiation region, including a "double" 35S CaMV promoter, the tomato fruit-specific E8 (E8) transcriptional initiation region, and those associated with T-DNA, such as the opine synthase transcriptional initiation region, e.g., octopine, mannopine, agropine, and the like.

Any one of number of regulatory sequences may be preferred in a particular situation, depending upon whether constitutive or tissue and/or timing induced transcription is desired, the efficiency of a particular promoter in conjunction with the heterologous SPS, the ability to join a strong promoter with a control region from a different promoter to provide for inducible transcription, ease of construction and the like. For example, tissue specific promoters can be employed to selectively modify or alter the composition of certain plant organs. Promoters which function in, or are specific by fruit, root and/or leaf are examples. These regulatory regions find ample precedence in the literature.

The termination region may be derived from the 3'-region of the gene from which the initiation region.was obtained, from the SPS gene, or from a different gene. Preferably the termination region will be derived from a plant gene, particularly, the tobacco ribulose biphosphate carboxylase small subunit termination region ; a gene associated with the Ti-plasmid such as the octopine synthase termination region or the tml termination region.

In developing the expression cassette, the various fragments comprising the regulatory regions and open reading frame may be subjected to different processing conditions, such a ligation, restriction, resection, *in vitro* mutagenesis, primer repair, use of linkers and adapters, and the like. Thus, nucleotide transitions, transversions, insertions, deletions, or the like, nay be performed on the DNA which is employed in the regulatory regions and/or open reading frame.

During the construction of the expression cassette, the various fragments of the DNA will usually be cloned in an appropriate cloning vector, which allows for amplification of the DNA, modification of the DNA or manipulation by joining or removing of the sequences, linkers, or the like. Normally, the vectors will be capable of replication in at least a relatively high copy number in *E. coli*. A number of vectors are readily available for cloning, including such vectors as pBR322, pUC series, M13 series, etc. The cloning vector will have one or more markers which provide for selection or transformants. The markers will normally provide for resistance to cytotoxic agents such as antibiotics, heavy metals, toxins, or the like. By appropriate restriction of the vector and cassette, and as appropriate, modification of the ends, by chewing back or filling in overhangs, to provide for blunt ends, by addition of linkers, by tailing, complementary ends can be provided for ligation and joining of the vector to the expression cassette or component thereof.

After each manipulation of the DNA in the development of the cassette, the plasmid will be cloned and isolated and, as required, the particular cassette component analyzed as to its sequence to ensure that the proper sequence has been obtained. Depending upon the nature of the manipulation, the desired sequence may be excised from the plasmid and introduced into a different vector or the plasmid may be restricted and the expression cassette component manipulated, as appropriate.

The manner of transformation of *E. coli* with the various DNA constructs (plasmids and viruses) for cloning is not critical to this invention. Conjugation, transduction, transfection or transformation, for example, calcium phosphate mediated transformation, may be employed.

In addition to the expression cassette, depending upon the manner of introduction of the expression cassette into the plant cell, other DNA sequences may be required. For example when using the Ti- or Ri-plasmid for transformation of plant cells, as described

below, at least the right border and frequently both the right and left borders of the T-DNA of the Ti- or Ri-plasmids will be joined as flanking regions to the expression cassette. The use of T-DNA for transformation of plant cells has received extensive study and is amply described in *Genetic Engineering, Principles and Methods* (1984) Vol 6 (Eds. Setlow and Hollaender) pp. 253-278 (Plenum, NY) ; A. Hoekema, in: *The Binary Plant Vector System* (1985) Offsetdrukkerij Ranters, 8.V. Alblasserdam.

Alternatively, to enhance integration into the plant genome, terminal repeats of transposons may be used as borders in conjunction with a transposase. In this situation, expression of the transposase should be inducible, so that once the expression cassette is integrated into the genome, it should be relatively stably integrated and avoid hopping.

The expression cassette will normally be joined to a marker for selection in plant cells. Conveniently, the marker may be resistance to a biocide, particularly an antibiotic, such as Kanamycin, G418, Bleomycin, Hygromycin, Chloramphenicol, or the like. The particular marker employed will be one which will allow for selection of transformed plant cells as compared to plant cells lacking the DNA which has been introduced.

A variety of techniques are available for the introduction of DNA into a plant cell host. These techniques include transformation with Ti-DNA employing *A. tumefaciens* or *A. rhizogenes* as the transforming agent, protoplast fusion, injection, electroporation, DNA particle bombardment, and the like. For transformation with Agrobacterium, plasmids can be prepared in *E. coli* which plasmids contain DNA homologous with the Ti-plasmid, particularly T-DNA. The plasmid may be capable of replication in Agrobacterium, by inclusion of a broad spectrum prokaryotic replication system, for example RK290, if it is desired to retain the expression cassette on a independent plasmid rather than having it integrated into the Ti-plasmid. By means of a helper plasmid, the expression cassette may be transferred to the *A. tumefaciens* and the resulting transformed organism used for transforming plant cells. Conveniently, explants may be cultivated with the *A. tumefaciens* or *A. rhizogenes* to allow for transfer of the expression cassette to the plant cells, and the plant cells dispersed in an appropriate selection medium. The *Agrobacterium* host will contain a plasmid having the *vir* genes necessary for transfer.

After transformation, the cell tissue (for example protoplasts, explants or cotyledons) is transferred to a regeneration medium, such as Murashige-Skoog (MS) medium for plant tissue and cell culture, for formation of a callus. Cells which have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.*, *Plant Cell Reports* (1986) 5:81-84. The transformed plants may then be analyzed to determine whether the desired gene product is still being produced in all or a portion of the plant cells. After expression of the desired product has been

demonstrated in the plant, the plant can be grown, and either pollinated with the same transformed strain or different strains and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited.

To identify the desired phenotypic characteristic, transgenic plants which contain and express a given SPS transgene are compared to control plants. Preferably, transgenic plants are selected by measurement of SPS activity in leaf, fruit and/or root. The SPS activity may be periodically measured from various stages of growth through senescence and compared to that of control plants. Plants or plant parts having increased or decreased SPS activity compared to controls at one or more periods are selected. Transgenic plants exhibiting SPS activity from about 1 to 12 fold that of control plants are preferred, with about 1 to 5 fold being more preferred, depending on a desired secondary trait. The activity can be compared to one or more other traits including SPS type, transcription initiation type, translation initiation type, termination region type, transgene copy number, transgene insertion and placement.

When evaluating a phenotypic characteristic associated with SPS activity, the transgenic plants and control plants are preferably grown under growth chamber, greenhouse, open top chamber, and/or field conditions. Identification of a particular phenotypic trait and comparison to controls is based on routine statistical analysis and scoring. Statistical differences between plants lines can be assessed by comparing SPS activity between plant lines within each tissue type expressing SPS. Expression and activity are compared to growth, development and yield parameters which include plant part morphology, color, number, size, dimensions, dry and wet weight, ripening, above- and below-ground biomass ratios, and timing, rates and duration of various stages of growth through senescence, including vegetative growth, fruiting, flowering, and soluble solid content including sucrose, glucose, fructose and starch levels. To identify transgenic plants having other traits, the plants can be tested for photosynthetic and metabolic activity, as well as end-product synthesis. For example, material isolated from transgenic plant cells and plant parts such as leaf, fruit and root are measured for end-products such as starch, sucrose, glucose, fructose, sugar alcohols, and glycine and serine from photorespiratory metabolism following standard protocols. Sweetness based on sugar content, particularly fructose, can be tested as well. For some plants, it may be necessary to modify growth conditions to observe the phenotypic effect. As an example, oxygen, carbon dioxide and light can be controlled and measured in an open gas chamber system, and carbon partitioning measured by C^{14} labeling of carbon dioxide or other metabolic substrates. Carbon partitioning also can be determined in extracts from fruit, leaf and/or root by chromatographic techniques or by Brix using a sugar refractometer. These characteristic also can be compared against or induced by growth conditions which vary

gas exchange parameters, light quality and quantity, temperature, substrate and moisture content between lines within each type of growing condition.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

Purification of Sucrose Phosphate Synthase of Corn

1.1 - Method of determination of enzymatic activity (SPS)

During purification SPS activity is followed in 2 ways:

a) either by means of a colorimetric test (Kerr *et al.*, *Planta.*, 1987, *170*:515-519) called resorcinol test described below.

Sucrose Phosphate Synthase catalyzes the reaction:

UDPG + Fructose 6-P <=> Sucrose 6-P + UDP

UDPG : Uridine Di-Phospho Glucose

Fructose 6-P or F6P : Fructose 6-Phosphate

Sucrose 6-P : Sucrose 6-Phosphate

The sucrose 6-P formed reacts with the resorcinol to give a red-colored compound quantifiable by spectrophotometry at 520 nm (nanometer) (Optical Density (O.D.) = 520 nm). In practice, to 45 µl (microliter) of enzymatic preparation 25 µl of a buffered solution containing the two substrates is added (UDPG 70 mM, F6P 28 mM, $MgCl_2$ 15 mM, HEPES 25 mM pH 7.5). After incubation at 37°C, the reaction is stopped by adding 70 µl of NaOH in solution and heating at 95°C during 10 min. After cooling, 0.25 ml of a solution 0.1% resorcinol in ethanol 95% is added; then 0.75 ml of HCl 30% is added. The OD at 520 mm is read after incubation for 8 min at 80°C, and cooling.

b) or by means of a coupled enzymatic system (Harbron *et al.*, *Anal. Biochem.* 1980, *107*:56-59) being composed in the following way:

UDPG	+	F6P < = > Sucrose 6-P		+ UDP
		SPS		
UDP	+	ATP < = > ADP		+ UTP
		Nucleoside Diphosphokinase NP_2K		
ADP	+	PEP < = > Pyruvate		+ ATP
		Pyruvate kinase PK		
Pyruvate	+	NADH < = > NAD	+ lactate	
		Lactate dehydrogenase LDH		

The disappearance of the NADH absorption at 340 nm is monitored: 1 mole of NAD formed or 1 mole of NADH consumed corresponds to 1 mole of sucrose 6 P formed.

In practice, in a quartz spectrophotometric tun thermostated at 37°C, the following solution are added.

- 540 µl of HEPES buffered 50 mM, $MgCl_2$ 10 mM, KCl 20 mM pH=7.5,
- 250 µl of a mixture of substrates PEP (1.6 mM NADH 0.6 mM, ATP 4 mM UDPG 112 mM),
- 60 µl of an enzyme mixture (LDH 166.7 U/ml PK 333.3 U/ml, NPzK 66.7 U/ml),
- 100 µl of F6P 112 mM.

After homogenization, 50 µl of the preparation containing SPS is added, the diminution of optical density at 340 nm is added with a spectrophotometer (UVIKON 860, KONTRON instruments). The measure is done with the kinetic of the machine.

1.2 Purification of the SPS (preparation of the immunogen)

1.2.1 Extraction

The starting material for the purification are nature leaves of young corn plants (*Zea mays* L. cv Pioneer 3184), which have been harvested in late morning, cut up, deveined, frozen in liquid nitrogen and stored at -70°C.

250 g of leaves are suspended in 1 liter of 50 mM HEPES 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, pH=7.5 buffer (extraction buffer) which has observed to it 11 g of Polyvinyl-pyrrolidone, nitrogen is bubbled through and the suspension is cooled to 0°C. The leaves are ground, until a homogeneous liquid is obtained. This ground product is filtered, and then centrifuged at 14,000 xg for 20 minutes at 4°C. While the bubbling through of nitrogen is maintained, a solution of 50% polyethylene glycol (PEG 8000 "Breox" at 50% w/v of extraction buffer) is added to the supernatant until a final concentration of PEG of 6% is reached. Then the suspension is cooled at 0°C. After

centrifuging at 14,000 g for 20 minutes the supernatant has added to it 50% PEG until a final concentration of PEG of 12% is reached. After a repeated centrifugation, the supernatant is discarded and the residue is solubilized with 60 ml of 50 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 10% ethylene glycol (EG), 0.08 M KCl, pH 7.5 buffer (recovery buffer). This solution is clarified by centrifuging at 40,000 g for 10 minutes. The supernatant constitutes the final extract.

1.2.2 Low pressure anion-exchange chromatography: fast-flow DEAE Sepharose exchanger

The final extract is chromatographed on a column 25 mm x 162 mm of 80 ml of Fast-Flow DEAE Sepharose (Pharmacia) equilibrated with recovery buffer. After washing the column with the same buffer, the proteins adsorbed on the support are eluted by means of a linear gradient with increasing ionic strength between 0.08 M KCl and 0.35 M KCl in the 50 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 10% EG, pH 7.5 buffer (buffer A). The flow rate applied during this experiment is 180 ml/h and chromatography is executed at 4°C.

The SPS activity is eluted at about 0.17 M KCl.

1.2.3 Chromatography on heparin Sepharose

The fractions containing the SPS activity are collected and diluted to one fifth in buffer A, then added to 12 ml of heparin Sepharose previously equilibrated with buffer A. After one hour of incubation with gentle agitation at 4°C, the gel is washed with about 10 volumes of buffer A + 0.05 M KCl, then repacked in a chromatography column.

The proteins adsorbed are eluted in an isocratic way by means of a 10 mM CAPS, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 10% EG, 0.01% Tween 80, 1 mg/ml heparin, 1% Fructose, 0.25 M KCl, pH 10 buffer, delivered at 60 ml/h. Chromatography is executed at 4°C. The fractions containing the SPS activity are collected (heparin fraction) and preserved on ice until the following purification stage. The enzyme at this stage is stable for a least one week.

The following purification steps are carried out using a system of High Performance Liquid Chromatography (HPLC) ; the purification is followed by means of a detector fitted with a filter enabling absorbency in the ultra-violet at 280 nm (A280) to be measured. The buffers and the fractions recovered are kept at low temperature.

1.2.4 High performance anion-exchange chromatography: Mono Q

The heparin fraction is diluted by adding one third volume of 20 mM

Triethanolamine, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, 3% EG, 0.3% Tween 80, pH 7.5 buffer (buffer A) and loaded on an FPLC Mono Q HR10/10 column, (10 x 100 mm Pharmacia) previously equilibrated with the same buffer which has added to it NaCl (final concentration 0.18 M). After the A280 has returned to 0, the proteins adsorbed on the chromatography support are eluted by means of a salt-complex gradient with buffer A (see above) and buffer B (buffer A + NaCl, 1 M) on a Mono Q column as shown below in Table 1.

Table 1

Salt Gradient for Mono Q Column

time (minutes)	% B
0	18
0.1	24
15	24
19	26
23	26
33	31
38	31
41	100
43	18

The flow rate applied to the Mono Q column is 180 ml/h. The SPS activity is eluted between 0.26 and 0.31 M NaCl. The active fractions are collected together ("Mono Q fraction").

1.2.5 HPLC on Hydroxyapatite

The Mono Q fraction is loaded on an HPLC column of hydroxyapatite 4 mm x 75 mm neutralized with 20 mM KH_2PO_4/K_2HPO_4, 3% EG, 0.3% Tween 80, 5 mM DTT, pH 7.5 buffer. After the A280 absorbance has returned to 0, the proteins adsorbed to the column are eluted by means of the following phosphate gradient using buffer A (see above) and buffer B (the same as buffer A additionally containing but 500 mM Phosphate of K) as shown below in Table 2.

Table 2

Phosphate Gradient for Hydroxyapatite Column

time (minutes)	% B
0	2
5	11
9	13
14	13
29	40
31	100
32	100
35	2

The flow rate applied to the column is 60 ml/h. At this stage, the phosphate will partially inhibit SPS activity and therefore it is difficult to calculate a specific activity and also a purification factor (see Table 1) at this stage. The SPS activity is eluted under these conditions with about 60 mM phosphate. The active fractions are collected together and constitute the HAC fraction.

1.2.6 HPLC on DEAE 5PW

The HAC fraction is loaded on an anion-exchange HPLC column of Di Ethyl Amino Ethyl type (DEAE-5PW) previously neutralized with a buffer of 20 mM Triethanolamine, 10 mM $MgCl_2$, 1 mM EDTA, 3% EG, 2.5 mM DTT, 2% betaine, pH 7.5 buffer (buffer A) + 0.15 M NaCl.

After the A280 absorbance has returned to 0, the proteins adsorbed to the column are eluted by means of the following NaCl gradient using buffer A (see above) and buffer B (the same as buffer A but additionally containing 1 M NaCl) as shown below in Table 3.

Table 3

Salt Gradient for DEAE Column

time (minutes)	% B
0	15
0.1	20
5	20
22	35
27	35
30	100
31	15

The flow rate applied to the column is 60 ml/h. The SPS activity is eluted with about 0.3M NaCl.

1.2.7 Preparation of the final preparation: concentration

The final preparation is concentrated by HPLC chromatography on a Mono Q HR5/5 exchanger (5 X 50 mm, Pharmacia) and rapid elution. The DEAE 5PW fraction (or the G200 fraction) is diluted to two thirds with buffer A (see 1.2.6) and loaded on the column which previously has been neutralized with buffer A + 0.18 M NaCl. The following gradient is then applied on the column using buffer A and B (see 1.2.6) as shown below in Table 4.

Table 4

Gradient for Concentration

time (minutes)	% B
0	18
10	40
12	100
13	18

The flow rate applied to the column is 60 ml/h. The SPS activity is eluted with about 0.3 M NaCl. The final preparation is stored at -20°C until used.

The results obtained at the various purification stages in terms of quantities of proteins recovered and of SPS activity are summarized in Table 5 below.

Table 5

Purification of Corn SPS

	Concentration of proteins (mg/ml)	Volume (ml)	sA[1] (ml)	pF[2]	Y[3] (%)
Ground product	1	1000	0.05	0	100
Final Extract	4< <8	60	0.30	6	144
DEAE FF fraction	0.4< <0.8	70	3	60	168
Heparin fraction	0.2< <0.4	25	9	180	90
Mono Q fraction	(0.02)[4]	30	-[5]	-[5]	-[5]
HAC fraction	(0.03)[4]	8	-[5]	-[5]	-[5]
Final preparation	0.05	2	25	500	5

[1]sA = Specific enzymatic activity: 1 U corresponds to 1 µmole of sucrose formed per minute per mg of protein at 37°C. The measurement of the quantity of proteins is carried out using the Bradford method. As Tween interferes enormously with this method, it is not possible to determine the proteins and then to calculate an sA at the level of the stages containing one. Furthermore, as phosphate is an inhibitor of SPS activity, the determination during the HAC stage gives an underestimated result.

[2]pF= Purification factor

[3]Y= Yield. The increasing yield during the initial stages of purification can be explained by the elimination, during purification, of certain inhibitors of SPS activity.

[4]( )= approximate value

[5]-= not determined

An SDS-PAGE profile at various stages of the purification process and the quality of the final preparation is given in Figure 1. The 120, 95 and 35 kd proteins are correlated to the SPS activity. The 35 and 95 kd proteins are very likely breakdown products of the 120 kd protein as it can be shown by the nucleotide sequence coding for the SPS protein. Furthermore, the antibodies directed against the 35 and 95 kd proteins also recognize the protein 120 kd in immunodetection after membrane transfer, which demonstrates an antigenic identity between these three proteins (see below). It must be pointed out, however, that the addition of protease inhibitors in the buffers during purification has not enabled us to obtain a single 120 kd protein.

Gel permeation chromatographies were carried out in order to determine the apparent molecular weight of the native SPS protein. Briefly, the HAC fraction was concentrated by HPLC chromatography on a Mono Q HR 5/5 inchanger (see 1.2.7). The active fractions were collected together (about 2 ml) and loaded on an G 200 column previously washed with a buffer containing 20 mM triethanolamine, 10 mM $MgCl_2$, 1 mM EDTA, 3% E.G., 2.5 mM DTT, 2% betain, 0.3 M NaCl pH 7.5. The SPS activity was eluted with a major protein peak corresponding to an apparent mass of 270-280 kda which is in agreement with the results obtained by Harbron *et al.* (*Arch. Biochem. Biophys.*, 1981, *212*:237-246) with the spinach SPS. It can be noted that the chromatography on a TS lambda 60000 permeation column lead to the elution of the SPS activity at a retention time corresponding to an apparent mass of 440 kda which is close to the value obtained by Doehlert and Huber (*Plant Physiol.*, 1983, *73*:989-994) with the spinach SPS, using an AcA34 permeation column.

The SPS protein seems therefore to be a di or tetrameric protein having as the basic sub-unit a 120 kda protein (homodimeric or homo-tetrameric). The results of SDS page analysis at various stages of purification are shown in Figure 1. The bands of proteins visible at about 120 kd (1), 95 kd (2) and 35 kd (3) are correlated, during the chromatography stages, with the appearance of SPS activity in the respective fractions.

Example 2

Process for the Preparation of Monoclonal Antibodies Directed Against SPS

2.1 Immunizations

BALB/c mice were immunized by subcutaneous injection (pads and paws) according to the following methodology: Day 0 injection of about 5 micrograms of proteins (or about 0.3 U SPS per mouse): Mono Q pool emulsified volume for volume with Freund's Complete Adjuvant (FCA).

Day 14 injection of about 5 micrograms of proteins (or about 0.3 U SPS per mouse): Mono Q pool emulsified volume for volume with Freund's Incomplete Adjuvant (FIA).

Day 27 Idem D14

Day 0 + 60 injection of about 20 micrograms of proteins: final pool in FIA

Day 0 + 90 injection of about 12 micrograms of proteins: final pool in FIA

Day 0 + 135 injection by intravenous route (IV) in the tail of about 20 micrograms of proteins: final pool.

Fusion is achieved 3 days after the IV immunization.

The sera were removed at D34, D61, D98 and D159 in order to measure the immune response (see screening).

2.1.1 Screening method

Two methods were used to detect antibodies specific to the SPS used for immunizations:

- detection method of antibodies inhibiting the SPS activity
- detection method of antibodies directed against the SPS (inhibiting or not).

a) Detection method of antibodies inhibiting the SPS activity

This method of screening allows the detection of antibodies which interfere with the active site of the SPS or on a site close to the latter, and therefore prevent the access of substrates. In practice, 70 µl of serum or of supernatant of hybridoma culture diluted in a suitable way was mixed with 70 µl of SPS preparation (Heparin fraction). After one hour of incubation at ambient temperature, the residual SPS activity was determined by coupled enzymatic determination (see 1.1). The results are expressed as a percentage of inhibition as compared to the same SPS preparation treated in the same way but without antibodies.

b) Detection method of antibodies directed against SPS (inhibiting or not)

This method is based on the precipitation of the antibody-SPS complex by goat anti-mouse IgG coupled to sepharose beads (GAM sepharose). In practice, 60 µl of serum or supernatant of hybridoma culture diluted in any suitable manner were added to 60 µl of SPS preparation (Heparin fraction). After 2 hours of incubation at ambient temperature, the mixture was added to 50 µl of 25% GAM-Sepharose previously washed three times with a buffer of 50 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 10% EG, 5 mM DTT, pH 7.5. The mixture was incubated overnight at 4°C with strong agitation. After centrifuging the mixture for 5 minutes at 3000 rpm, the residual SPS activity in the supernatant was determined by coupled enzymatic determination (see 1.1). The results are expressed as a percentage of precipitation (% prec.) as compared to the same SPS preparation treated in the same way without antibodies.

2.1.2 Results

10 mice were immunized according to the protocol described previously. The following table gives the results of the precipitation determinations carried out with the heteroantisera of the 10 mice on D159. The sera are diluted to one two-hundredth.

Table 6

Percentage Precipitation of Antibody-SPS Complex

Mouse	1	2	3	4	5	6	7	8	9	10
% Prec.	45	22	32	64	36	30	22	16	39	37

Additional dilutions of the serum of mouse 4 give the following results:

Table 7

Percentage Precipitation of Serial Dilutions of Mouse 4 Serum

Dilution	% Precipitation
1/200	67
1/400	48
1/600	29
1/1000	20

The spleens of mice 1 and 4 were used for the fusion with myeloma cells.

2.2 Cellular fusion

The splenocytes of the mice were fused with myeloma cells of SP2/0-Ag14 mice according to a ratio of 2:1 in the presence of 45% polyethylene glycol 1500. The selection of the hybridomas was effected by adding hypoxanthine and azaserine to the culture medium 24 and 48 hours after fusion.

The hybridomas were cloned and sub-cloned by the method of limited dilution.

2.2.1 Results of the screening of hybrids and clones

Results from screening of hybrids, clones and sub-clones are shown below in Table 8.

Table 8

Hybrid, Clone and Sub-clone Screening

Hybrids

Mouse 4 (SPA fusion)	Mouse 1 (SPB fusion)
2 positive hybrids out of 45	6 positive hybrids out of 52
SPA2 : 38 % prec.	SPB3 : 17 % prec.
SPA19: 7 % prec.	SPB5 : 67 % prec.
	SPB8 : 53 % prec.
	SPB13 : 68 % prec.
	SPB25 : 13 % prec.
	SPB34 : 17 % prec.

Clones

SPA fusion	SPB fusion
2 clones retained out of 36	7 clones retained out of 46
SPA2-2 : 85 % prec.	SPB3-2 : 19 % prec.
SPA19-7 : 8 % prec.	SPB5-1 : 76 % prec.
	SPB5-2 : 71 % prec.
	SPB5-3 : 45 % prec.
	SPB5-4 : 24 % prec.
	SPB13-1 : 79 % prec.
	SPB13-2 : 53 % prec.

Sub-Clones

SPA fusion	SPB fusion
sub-clones retained out of 48	sub-clones retained out of 72
SPA2-3 : 60 % prec.	SPB3-2-19 : 21 % prec.
SPA2-2-33 : 33 % prec.	SPB5-2-10 : 86 % prec.
SPA2-2-25 : 92 % prec.	SPB5-4-2 : 46 % prec.
	SPB13-1-7 : 87 % prec.
	SPB13-2-2 : 93 % prec.

2.2.2 Production of anti-SPS monoclonal antibodies

The hydridomas were injected by the intra-peritoneal route into female BALB/c mice previously treated with pristane. The monoclonal antibodies were partially purified from ascites fluids precipitated with 18% sodium sulphate. The proteins so precipitated were dissolved then dialyzed against PBS (F18).

2.2.3 Characterization of anti-SPS monoclonal antibodies

a) Typing

The typing was done using an ELISA test. Anti-IgG rabbit and anti-IgM mouse

antibodies (Zymed) were fixed at the bottom of the wells of a 96-well plate. After one night at ambient temperature the unoccupied sites were saturated with a solution of 3% bovine serum albumin in PBS. After one hour of incubation at 37°C and several washes, the various F18's were deposited in the wells. After incubation and several washes, goat or rabbit antibodies, anti-class and anti-sub class mouse immunoglobulins linked with peroxidase, were added. After one hour at 37°C, the antibody type was identified using an H_2O_2/ABTS system. All the anti-SPS monoclonal antibodies were found to be of IgG_1 type.

b) Inhibition of SPS activity

The determination of the capacity of the antibodies to inhibit the SPS activity was carried out by the technique mentioned previously (see 2.1.1 a) using F18's. The results are shown below in Table 9.

Table 9

Inhibition of SPS Activity

Antibody	Concentration of antibodies (mg/ml)	% Inhibition
SPA2-2-3	50	0
SPA2-2-22	50	0
SPA2-2-25	50	0
SPA3-2-19	50	0
SPA5-2-10	50	0
SPA5-4-2	50	0
SPA13-1-7	50	50
	25	55
	5	25
	2.5	10
	1	2.1
SPB13-2-2	50	60.1
	25	59.1
	5	33.8
	2.5	14.2
	1	8.7

c) Immuno-precipitation of the SPS activity

The determination of the ability of the antibodies to immunoprecipitate the SPS activity was carried out by the technique mentioned previously (see 2.1,1 b) using F18's. The results are shown below in Table 10.

Table 10

Immunoprecipitation of SPS Activity

Antibody	Concentration of antibodies (mg/ml)	% Precipitation
SPA2-2-3	50	95
	25	92
	5	80
	2.5	40
	1	20
SPA2-2-22	50	95.7
	25	95
	10	51
	5	48.2
	2.5	25
	1	10.1
SPA2-2-25	50	91.3
	25	95.3
	5	90.4
	2.5	22.8
	1	12.5
SPB3-2-19	50	95
	25	95
	5	27.8
	2.5	17.8
	1	9.3
SPB5-2-10	50	95
	25	95
	5	81.1
	2.5	41.4
	1	22.6
SPB5-4-2	50	95
	25	95
	5	86.1
	2.5	57.2
	1	26.1
SPB13-1-7	50	95
	25	95
	10	65.4
	5	48.1
	2.5	15
	1	10
SPB13-2-2	50	95
	25	95
	5	71.8
	2.5	43.5

Example 3

Use of the Monoclonal Antibodies for the Characterization and Purification of SPS

3.1 Characterization of Corn SPS

This characterization was carried out with SPB3-2-19 and SPB13-2-2 antibodies by the technique of immuno-detection after transfer of the proteins from an electrophoresis gel under denaturing conditions (SDS-PAGE) on nitrocellulose membrane (Western). After electrophoretic separation in a 12.5% acrylamide gel (Nature 277 (1970) 680-685), the proteins were transferred onto a 0.22 µm nitrocellulose membrane (Schleicher and Schuell). The buffer was a standard electrophoresis buffer (3.03 g/l. TRIS base, 14.4 g/l. Glycine, 0.1% SDS, pH 8.3, 20% methanol).

After transfer, the membrane was put in a blocking bath (0.5% Casein in PBS). After one hour at 37°C under gentle agitation, the membrane was washed 3 to 4 times in a washing buffer (0.1% Casein, 0.5% Tween 20, in PBS) then incubated with a solution of 10 micrograms/ml of the monoclonal antibody to be tested. A part of the membrane was incubated in parallel with a non-immune antibody (negative control). After one hour of incubation at ambient temperature followed by 9 or 10 washes, the membrane was incubated in the presence of an anti-mouse antibody labeled with ^{125}I diluted in a washing buffer (50,000 cpm per cm^2 of membrane). After one hour of incubation at ambient temperature followed by 9 or 10 washes, the membrane was dried, then autoradiographed (X-OMAT AR Kodak film and Crone XTRA Life Dupont amplifying screen). The results of the autoradiography are shown in Figure 2. In the autoradiograph, a strong signal is observed at the protein bands 120 kd, 95 kd and 35 kd which correlates with the previous results (see first part).

3.2 Purification of Sucrose Phosphate Synthase by Immunoaffinity Chromatography

A methodology for the purification of corn Sucrose Phosphate Synthase on an immunoaffinity support has been perfected in order to increase the quantity of protein recovered while reducing the number of purification stages and to obtain quantities sufficient for protein sequencing.

3.2.1 Preparation of the immuno-adsorbent

The F18 (see 2.2.2) corresponding to the SPB13-1-7 antibody or to the SPB13-2-2 antibody were mixed with activated CH-Sepharose, (1 mg of antibody per ml of gel). After incubation for 2 hours at ambient temperature, the sites not occupied by the antibodies were saturated with 1M ethanolamine, pH 9. The support was then washed

alternately with O.1M acetate, 0.5 M NaCl, pH 4 buffer and 0.1 M TRIS, 0.5 M NaCl, pH 8 buffer. The immunoaffinity support thus prepared was preserved at 4°C in a 50 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, zero 0.01% sodium nitride (azide), pH 7.5 buffer.

3.2.2 Immunoaffinity Chromatography

50% PEG was added to the Heparin fraction of SPS (see 1.2.3.) to give a final concentration of PEG of 20%. After incubation for 30 minutes at 4°C with gentle agitation, the mixture was centrifuged at 1600 g for 30 minutes. The protein deposit was taken up in half of the initial volume with the 50 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 10% ethylene glycol, pH 7.5 buffer. This stage allows the previous buffer, which is incompatible with the immunoaffinity chromatography, step to be eliminated, and the proteins to be concentrated. The yield of SPS activity was from 80 to 90%.

The solution obtained was applied with a flow rate of 0.1 ml/min over 1 ml of immunoaffinity support packed in a column and on which had been fixed an antibody not directed against the SPS (activated CNBr-Sepharose, on which an antineomycin antibody is fixed). This first stage allows the elimination of certain contaminants which are fixed nonspecifically on the chromatography support. The effluent of the non-specific column was in turn applied to the anti-SPS immunoaffinity support (2 ml in an 11 x 20 mm column) with a flow rate of 0.1 ml/min. These two stages were carried out at laboratory temperature. The column was washed with 10 ml of load buffer and then with a washing buffer (load buffer with the addition of 0.25 M NaCl and 0.3% Tween 20) until absorbency in ultra-violet at 280 nm was close to base level. The proteins adsorbed on the support were eluted with a solution of 50 mM triethylamine, pH 11. This elution was carried out at 4°C and the immunoaffinity column was reversed to obtain an optimum yield. The SDS-PAGE profile of the final preparation obtained corresponds to that obtained using the standard protocol (see 1). It must be noted that the elution method of the proteins adsorbed on the immunoaffinity support irreversibly destroys the SPS activity but the recovery yield of the eluted SPS proteins is optimal compared to tests carried out in native elution conditions. The eluate of the immunoaffinity column was desalted using a Sephadex G25 column, against a 0.14% Glycerol, 0.07% 2-mercapto-ethanol, 0.04% SDS, 0.9 mM TRIS pH 6.8 buffer (electrophoresis buffer in reducing conditions diluted 70 times). After desalination, the protein preparation was concentrated 70 times with a concentrator under vacuum and the SPS proteins were purified by SDS-PAGE (see below).

Example 4

Partial Sequencing of SPS Polypeptides

4.1 Purification of SPS Polypeptides for Sequencing

Samples of a purified protein preparation obtained as described in Example 3.2.2. were subjected to preparative SDS-PAGE. After electrophoresis, the protein bands were visualized with KCl treatment as described by Bergman and Joernvall (*Eur. Biochem.* (1978) *169*:9-12) and the bands observed at 90kd and 30kd were excised. The proteins from these gel fragments were electroeluted using an Electrophoretic Concentrator according to manufacturer's instructions (ISCO ; Lincoln, NE) in 4 mM sodium acetate, pH8. After electroelution, protein yields were quantitated by comparison to a bovine serum albumin (BSA) standard on a Comassie Blue-stained gel. Approximately 30 mg of the 30 kd protein and 75 μg of the 90 kd protein were obtained.

4.2 Tryptic Digestion and Protein Sequencing of SPS polypeptides

The proteins were concentrated by acetone precipitation, and resuspended in 50 mM ammonium carbonate buffer, pH 8. Tryptic digestion and HPLC purification were performed as described by Sturm and Chrispeels (*Biol. Chem.* (1987) *262*:13392-13403). Briefly, digestion was performed by addition of trypsin (5% of SPS protein), and incubation for two hours at 37°C. The digestion was then repeated. The proteins were concentrated by lyophilization and resuspended in 50mM sodium phosphate buffer, pH 2.2. This mixture was subjected to reverse phase HPLC separation by application to a C18 column in phosphate buffer. Elution was performed using an increasing gradient of acetonitrile. Eluted material from the phosphate buffer/acetonitrile gradient was monitored at 214 nm. The fractions corresponding to peaks of absorbance at 214 nm were collected, lyophilized, resuspended in 0.1% trifluoroacetic acid, reapplied to the C18 column (equilibrated with 0.1% trifluoroacetic acid), and eluted using an acetonitrile gradient. Eluted material from the trifluoroacetic acid/acetonitrile gradient was monitored at 214 nm. The fractions corresponding to peaks of absorbance at 214 nm were collected, lyophilized, and subjected to standard Edman degradation protein sequencing on an automated protein sequencer (Applied Biosystems ; Foster City, CA). Sequences of five peptides were obtained. See Fig. 3 (SEQ ID NOS: 1-5).

Example 5

Isolation and Assembly of a Full-Length cDNA for SPS

5.1 RNA Isolation from Corn Leaf

Total RNA was isolated from corn leaves (see 1.2.1.) according to the method of Turpen and Griffith (*Biotechniques* (1986) *4*:11-15). Briefly, 250 gm of material was homogenized in 4M guanidine thiocyanate and 2% sarcosyl. The mixture was then centrifuged and the cleared supernatant was layered into a 5.7 M CsCl cushion and centrifuged for 5.5 hours at 50,000 rpm. The RNA pellet was dissolved in water, extracted with phenol and chloroform, and precipitated with ethanol. The resulting pellet was resuspended in water. The final yield from the RNA isolation step was quantitated by UV spectrophotometry.

5.2 Poly(A) RNA Isolation

A saturated suspension of cellulose powder/water was added to the RNA/water mixture obtained in 5.1, at 10% of the total volume, to remove residual polysaccharides. After centrifugation, the supernatant, containing the RNA, was applied to an oligo(dT)-cellulose column as described by Maniatis *et al.* (*Molecular Cloning: A Laboratory Manual*, (1982) Cold Spring Harbor, New York). The fraction containing the poly(A)+ RNA was then reapplied to the column. The eluted fraction containing the poly(A)+ RNA was extracted with phenol, and the RNA was precipitated with ethanol. Analysis by gel electrophoresis showed complete absence of ribosomal RNA.

5.3 Construction of Total Corn Leaf Library

cDNA synthesis was performed according to the manufacturer's instructions (RiboClone cDNA Synthesis System by Promega, Madison, WI), using five μg of poly(A)+ RNA as template, except that M-MLV reverse transcriptase (BRL ; Bethesda, MD) was substituted for AMV reverse transcriptase. *Eco*RI linkers were added to the blunt-ended cDNA, and the resulting fragments were cloned into an expression vector (LambdaZAP, Stratagene ; La Jolla, CA) according to the manufacturer's instructions. The resulting library contained approximately 1.5×10^6 transformants.

5.4 PCR Generation of a Partial SPS cDNA Probe

Using the sequence information from the peptides of Example 4 (SEQ ID NOS: 8-9) and the polymerase chain reaction (PCR), a 1200 bp SPS cDNA fragment was generated. Total corn leaf cDNA (5.3.) was used as a template, and degenerate oligonucleotides (SEQ ID NOS: 10-13), designed from two peptide sequences of the 30kd and 90kd SPS

polypeptides, were used as primers. These primer sets were designated as CD3 (SEQ ID NOS: 10-11) and CD4 (SEQ ID NOS: 12-13). See Fig. 4. PCR was carried out, according to the manufacturer's instructions (GeneAmp DNA Amplification Reagent Kit and DNA Thermal Cycler of Perkin Elmer Cetus ; Norwalk, CT) except that the reaction was carried out for 30 cycles, and the annealing steps were programmed to be at 50°C for 1 minute. The PCR reactions were analyzed by agarose gel electrophoresis. Use of the correct set of primers, CD3, resulted in a 1200 bp band being generated by the PCR reaction. PCR using the other set of primers, CD4, gave no specific signals. See Fig. 5. Southern analysis (see Fig. 5) confirmed that the PCR band was not an artifact. The probe 4K5 (SEQ ID NO: 14) was used because the corresponding sequence of the probe was predicted to be within the 1200bp fragment if the fragment corresponded to the SPS sequence. The probe hybridized to the 1200 bp band generated by PCR using the primer set CD3 but not to PCR products generated by the primer set CD4. See Fig. 5.

5.5 Isolation of SPS Bacteriophage Lambda cDNA Clones

The 1200 bp PCR-generated fragment was labeled with ^{32}P (as per the Random Primed DNA Labeling Kit, Boehringer Mannheim, Indianapolis, IN) and used as a probe to screen approximately 250,000 plaques of the cDNA library (5.3.). The inserts of the positive clones were analyzed by restriction analysis with *Eco*RI, and the clones with the longest inserts, SPS#3 and SPS#18, were selected for further analysis. See Fig. 6. A 0.4 kb *Hin*dIII/*Eco*RI fragment from the 5' end of SPS#3 was isolated, then labeled with ^{32}P by random priming (Random Primed DNA Labeling Kit) and used as a probe to re-screen the library. Another clone, designated SPS#61, which extends further upstream than SPS#3, was isolated. See Fig. 6. DNA sequencing indicated that the 5' end of the SPS reading frame was not reached.

To isolate cDNA clones that included more of the 5' region than SPS#3 or SPS#61, a new cDNA library was prepared, as per Example 5.3., (RiboClone cDNA Synthesis System by Promega ; Madison, WI) using M-MLV reverse transcriptase instead of AMV reverse transcriptase. However, instead of using oligo (dT) as a primer, a synthetic 17 bp primer, 23B, derived from the 5' sequence of the SPS#61 clone, was used (see Fig. 6). This resulted in cDNAs that contain only regions upstream of the SPS#61 5' region. The library was screened with the ^{32}P-labeled *Eco*RI insert from SPS#61, and 16 positive clones were obtained. The clones with the longest inserts, SPS#77 and SPS#90, were selected for further analysis. DNA sequencing of SPS#77 and SPS#90 showed that the region of overlap (greater than 100 bp) with SPS#61 was identical in all clones, and that both extended further upstream into the 5' region. See Fig. 6.

PCR was carried out using single-stranded cDNA (from a reverse transcriptase reaction corn leaf RNA (5.2.) primed with oligo (dT) as described above) as template and primers selected from the SPS#90 and SPS#3 sequences, confirmed that SPS#90 and SPS#3 originate from the same mRNA transcript. The fragment resulting from this PCR reaction was 750 bp in length, consistent with the size predicted from the DNA sequence. The 750 bp fragment was subcloned into a Bluescript-derived vector as a *SalI*/*Hin*dIII fragment. Four of the resulting subclones were partially sequenced, and the sequence obtained matched the existing DNA sequence.

5.6 Assembly of the SPS Reading Frame

Both DNA strands of SPS#90, SPS#61, and SPS#3 were sequenced, using the method of Sanger *et al.* (*PNAS* (1977) *74*:5463-5467). All three sequences can be combined to form one contiguous sequence of 3509 bp. See Fig. 7 (SEQ ID NO: 6). Primer extension experiments using corn leaf poly(A) RNA and an antisense primer showed that the 5' end of our DNA sequence represents sequences form the actual 5' end of the SPS in RNA. In the SPS reading frame, as defined by the five peptide sequences (SEQ ID. NOS.: 1-5 respectively) (see Fig. 3), the first methionine codons are located at bp 112 and bp 250. See Fig. 7 (SEQ ID NO: 6). The codon at bp 112 is similar to the consensus eukaryotic translational start site (Kozak, *Cell* (1986) *44*:283-292) and is located 54 bp downstream of a TAG stop codon (bp 58). It is proposed that this codon represents the translational start of the SPS polypeptide *in vivo*. After a 1068 codon reading frame, translation is stopped by TGA. The following 193 bp contain the 3' untranslated region including a poly(A) addition signal, AAATAAA.

The full-length SPS coding region can be assembled by combining the 529 bp *Bam*HI/*Hin*dIII fragment of SPS#90, the 705 bp *Hin*dIII fragment of SPS#61 and the 2162 bp *Hin*dIII/ *Eco*RI fragment from SPS#3 (see Fig. 6).

Example 6

Detection of SPS Polypeptides by Specific Antisera

6.1 Preparation of Antibodies to SPS

Samples of purified protein preparations obtained by the method described in 3.2.2. were subjected to SDS-PAGE electrophoresis. The proteins in the gel were fixed and stained. The bands corresponding to the 90kd and 30kd polypeptides were excised. Using

this material, polyclonal antisera were raised in rabbits by conventional procedures. Western analysis (as described by Oberfelder, *Focus* (1989) *11(1)*:1-5) showed that the antibodies isolated from the rabbit immunized with SPS 30 recognized the bands corresponding to the SPS#30 and SPS#120 peptides on a SDS PAGE gel, and that the antibodies isolated from the rabbit immunized with SPS#90 recognized the bands corresponding to the SPS#90 and SPS#120 polypeptides (see Fig.8).

6.2 Immunological Localization of SPS in the Corn Plant

Total proteins were extracted from leaves of a 30 day-old corn plant, harvested at 11:00 am, by boiling in SDS buffer. The protein extracts were loaded on duplicate SDS-PAGE gels. One gel was stained with Comassie Blue, while the other was subjected to Western analysis, using a mixture of SPS#30 and SPS#90 antisera as probe. See Fig. 9. The prominent bands appearing on the Comassie Blue-stained gel were identified as phosphoenolpyruvate carboxylase (PEPcase), an enzyme involved in C4 photosynthesis. The Western blot showed the presence of the SPS band. The SPS protein pattern was very similar to the PEPcase protein pattern: not present in roots, nor present in the section of leaf closest to the stem, nor present in very young leaves. This pattern corresponds with expression associated with photosynthesis, and is the pattern expected for SPS.

Example 7

Construction of Expression Construct Plasmids

7.1 Construction of the full-length SPS reading frame

Clone SPS#90 was digested with *Hin*dIII and ligated with the 705 bp *Hin*dIII fragment from clone SPS#61 to create a plasmid containing the 5' end of the SPS coding region. The resulting plasmid was digested with *Bam*HI and partially digested with *Hin*dIII, resulting in a 1340 bp *Bam*HI/*Hin*dIII fragment containing the 5' end of the coding region. The 3' end of the SPS coding region was obtained by digestion of SPS#3 with *Eco*RI and partial digestion with *Hin*dIII, resulting in a 2162 bp *Hin*dIII/*Eco*RI fragment. This 2162 bp *Hin*dIII/*Eco*RI fragment, carrying the 3' end, was ligated with the 1340 *Bam*HI/*Eco*RI fragment carrying the 5' end into a *Bam*HI/*Eco*RI-digested pUC-derivative plasmid Bluescript, to create a plasmid carrying the entire 3403 bp SPS coding region and 3' untranslated transcription termination region.

7.2 Expression of SPS in *E. coli*

When cloning the 3403 bp *Bam*HI/*Eco*RI SPS fragment into the plasmid Bluescript-SK (Stratagene, La Jolla, CA), a translational fusion between the plasmid coded *lac*Z sequence and the SPS reading frame was created. The resulting fusion protein contains 30 N-terminal amino acids from the ß-galactosidase and the complete SPS polypeptide. The fusion protein was expressed in *E. coli* under the Bluescribe plasmid *lac*Z promoter. Preparation of total protein followed by Western analysis using anti-SPS antisera (see 6.1.) shows a band comigrating with native plant SPS. For the SPS activity test, the *E. coli* cells containing the SPS expression construct as described were opened with lysozyme and sonication. Soluble protein was desalted by a Sephadex G-25 column. This protein extract was assayed for SPS activity analogous to the method described in l.l.a., except that the reagent anthrone was used instead of resorcinol (Handel, *Analytical Biochemistry*, (1968) *22*:280-283). This test showed that the SPS protein, expressed from the cDNA in *E. coli* does have SPS enzyme activity. By comparison to native plant enzyme it seems to have the same specific activity.

7.3. Construction of the Tobacco Small Subunit (SSU) Promoter-Transcriptional Fusions

The SPS coding region can be conveniently cloned as a *Bam*HI/*Eco*RI (bp 106 - bp 3506) fragment 3' of a tobacco small subunit promoter. A SSU promoter for expression of the SPS coding region, was prepared as follows. The SSU promoter region from pCGN627 (described below) was opened by *Kpn*I and the 3' overhang removed. After *Eco*RI digestion, the 3403 bp *Bam*HI (filled in) *Eco*RI SPS cDNA fragment (see, Example 7.1.) was inserted. After the SPS coding region was ligated into the SSU promoter, the SSU/SPS region was ligated into a binary vector and integrated into a plant genome via Agrobacterium tumefaciens-mediated transformation. (The SPS region carries its own transcription termination region in the cDNA sequence). Insertion of the SSU/SPS construct into the binary vector pCGN1557 resulted in pCGN3812.

pCGN627

The 3.4 kb *Eco*RI fragment of TSSU3-8 (O'Neal *et al.*, *Nucleic Acids Res.* (1987) *15*:9661-8677), containing the small subunit promoter region, was cloned into the *Eco*RI site of M13mpl8 (Yanisch-Perron et al, *Gene* (1985) *53*:103-119) to yield an Ml3 clone 8B. Single-stranded DNA was used as a template to extend the oligonucleotide primer "Probe

1" (O'Neal *et al.*, *Nucleic Acids Research* (1987) *15*:8661-8677) using the Klenow fragment of DNA polymerase I. Extension products were treated with mung bean nuclease and then digested with *Hind*III to yield a 1450 bp fragment containing the SSU promoter region. The fragment was cloned into *Hind*III-*Sma*I-digested pUC13 (Yanisch-Perron *et al.*, *Gene* (1985) *53*:103-119) to yield pCGN625. pCG2J625 was digested with *Hind*III, the ends blunted with Klenow, and the digested plasmid re-digested with *Eco*RI. The *Eco*RI/blunted-*Hind*III fragment containing the SSU promoter region was ligated with *Sma*I/*Eco*RI-digested pUC18 to yield pCGN627.

7.4. Construction of a CaMV Promoter--SPS Transcriptional Fusion

The 35ESS promoter-DNA fragment from cauliflower mosaic virus was fused to the SPS DNA as follows. The plasmid pCGN639 was opened by *Bam*HI and *Eco*RI and the 3403 bp *Bam*HI-*Eco*RI SPS cDNA fragment (described in Example 7.1) was cloned into this plasmid. The hybrid gene was removed from this plasmid as a 4.35 kb *Xba*I-*Eco*RI fragment and ligated into a binary vector (McBride and Summerfelt, *Plant Mol. Bio.* (1990) *14*:269-276) and integrated into a plant genome via *Agrobacterium tumefaciens* mediated transformation. Insertion of the CaMV/SPS construct into the binary vector pCGN1557 (McBride and Summerfelt *supra*) results in pCGN3815.

7.4.1. Construction of pCGN639

pCGN164 was digested with *Eco*RV and *Bam*HI to release a *Eco*RV-*Bam*HI fragment which contained a portion of the 35S promoter (bp 7340-7433). pCG8638 was digested with *Hind*III and *Eco*RV to release a *Hind*III-*Eco*RV fragment containing a different portion of the 35S promoter (bp 6493-7340). These two fragments were ligated into pCGN986 which had been digested with *Hind*III and *Bam*HI to remove the *Hind*III-*Bam*HI fragment containing the 35S-promoter ; this ligation produced pCGN639, which contains the backbone and tml-3' region from pCGN986 and the two 35S promoter fragments from pCGN164 and pCGN638.

7.4.2. Construction of pCGN164

The *Alu*I fragment of CaMV (bp 7144-7735) (Gardner *et al.*, *Nucl. Acids Res.* (1981) 9:2871-2888) was obtained by digestion with *Alu*I and cloned into the *Hin*cII site of M13mp7 (Vieira and Messing, *Gene* (1982) *19*:259-268) to create C614. An *Eco*RI digest

of C614 produced the *Eco*RI fragment from C614 containing the 35S promoter which was cloned into the *Eco*RI site of pUC8 (Vieira and Messing, *supra*) to produce pCGN146. To trim the promoter region, the *Bgl*II site (bp 7670) was treated with *Bgl*II and *Bal*31 and subsequently a *Bgl*II linker was attached to the *Bal*31 treated DNA to produce pCGN147. pCGN147 was digested with *Eco*RI/*Hph*I and the resulting *Eco*RI-*Hph*I fragment containing the 35S promoter was ligated into *Eco*RI-*Sma*I digested M13mp8 (Vieira and Messing, *supra*) to create pCGN164.

7.4.3. Construction of pCGN638

Digestion of CaMV10 (Gardner, *et al.*, *Nucl. Acids Res.* (1981) 9:2871-2888) with *Bgl*II produced a *Bgl*II fragment containing a 35S promoter region (bp 6493-7670) which was ligated into the *Bam*HI site of pUC19 (Norrander *et al.*, *Gene* (1983) 26:101-106) to create pCGN638.

7.4.4. Construction of pCGN986

pCGN986 contains a cauliflower mosaic virus 35S (CaMV35) promoter and a T-DNA tml-3' region with multiple restriction sites between them. pCGN986 is derived from another cassette, pCGN206, containing a CaMV35S promoter and a different 3' region, the CaMV region VI 3'-end and pCGN971E, a tml 3' region. pCGN148a containing a promoter region, selectable marker (kanamycin with 2 ATG's) and 3' region, was prepared by digesting pCGN528 with *Bgl*II and inserting the *Bam*HI-*Bgl*II promoter fragment from pCGN147 (see 7.4.2. above). This fragment was cloned into the *Bgl*II site of pCGN528 so that the *Bgl*II site was proximal to the kanamycin gene of pCGN528.

The shuttle vector used for this construct pCGN528, is made as follows: pCGN525 was made by digesting a plasmid containing Tn5, which harbors a kanamycin gene (Jorgensen *et al.*, *Mol. Gen. Genet.* (1979) 177:65), with *Hin*dIII-*Bam*HI and inserting the *Hin*dIII-*Bam*HI fragment containing the kanamycin resistance gene into the *Hin*dIII-*Bam*HI sites in the tetracycline gene of pACYC184 (Chang and Cohen, *J. Bacteriol.* (1978) 134:1141-1156). pCGN526 was made by inserting the *Bam*HI fragment 19 of pTiA6 (Thomashow *et al.*, *Cell* (1980) 19:729-739) modified with *Xho*I linkers inserted into the *Sma*I site, into the *Bam*HI site of pCGN525. pCGN528 was obtained by deleting the small *Xho*I and religating.

pCGN149a was made by cloning the *Bam*HI kanamycin gene fragment from pMB9KanXXI into the *Bam*HI site of pCGN148a. pMB9KanXXI is a pUC4K variant (Vieira and Messing, *Gene* (1982) *19*:259-268) which has the *Xho*I site missing but contains a functional kanamycin gene from Tn903 to allow for efficient selection in Agrobacterium.

pCGN149a was digested with *Hin*dIII and *Bam*HI and ligated which pUC8 (Vieira and Messing, *supra*) digested with *Hin*dIII and *Bam*HI to produce pCGE169. This removes the Tn9O3 kanamycin marker. pCGN565 and pCGN169 were both digested with *Hin*dIII and *Pst*I and ligated to form pCGN203, a plasmid containing the CaMV 35S promoter and part of the 5'-end of the Tn5 kanamycin gene (up to the *Pst*I site, (Jorgensen *et al.*, *Mol. Gen. Genet.* (1979) *177*:65). pCGN565 is a cloning vector based on pUC8-Cm (K. Buckley, Ph.D. Thesis, UC San Diego 1985), but containing the polylinker from pUC18 (Yanisch-Perron *et al.*, *Gene* (1985) *53*:103-119). A 3' regulatory region was added to pCGN203 from pCGN204 (an *Eco*RI fragment of CaMV (bp 408-6105) containing the region VI 3' cloned into pUC18 (Gardner *et al.*, *Nucl. Acids Res.* (1981) *9*:2871-2888) by digestion with *Hin*dIII and *Pst*I and ligation. The resulting cassette, pCGN206, is the basis for the construction of pCGN986.

The pTiA6 T-DNA tml 3'-sequences were subcloned from the *Bam*l9 T-DNA fragment (Thomashow *et al.*, *Cell* (1980) *19*:729-739) as a *Bam*HI-*Eco*RI fragment (nucleotides 9062 to 12,823, numbering as in Barker *et al.*, *Plant Mol. Biol.* (1983) 2:335-350) and combined with the pACYC184 (Chang and Cohen, *J. Bacteriol.* (1978) *134*:1141-1156) origin of replication as an *Eco*RI-*Hin*dII fragment and a gentamycin resistance marker (from plasmid pLB41), (D. Figurski) as a *Bam*HI-*Hin*dII fragment to produce pCGN417. The unique *Sma*I site of pCGN417 (nucleotide 11,207 of the *Bam*l9 fragment) was changed to a *Sac*I site using linkers and the *Bam*HI-*Sac*I fragment was subcloned into pCGN565 to give pCGN971. The *Bam*HI site of pCGN971 was changed to an *Eco*RI site using linkers to yield pCGN971E. The resulting *Eco*RI-*Sac*I fragment of pCGN971E, containing the tml 3' regulatory sequence is joined to pCGN206 by digestion with *Eco*RI and *Sac*I to give pCGN975. The small part of the Tn5 kanamycin resistance gene was deleted from the 3'-end of the CaMV 35S promoter by digestion with *Sal*I and *Bgl*II, blunting the ends and ligating with *Sal*I linkers. The final expression cassette, pCGN986, contains the CaMV 35S promoter followed by two *Sal*I sites, an *Xba*I site, *Bam*HI, *Sma*I, *Kpn*I sites and the tml 3' region (nucleotides 11207-9023 of the T-DNA).

A schematic summary of the construction of the various plasmids is shown in Figures 10A through 10C.

Example 8

Transgenic SPS Tomato Plants

8.1 Production of Tissue-Specific SPS "Sense" Transgenic Tomato Plants

Tomato plants were transformed with expression cassettes containing SPS encoding sequences (pCGN3812, pCGN3815, pCGN3342, and pCGN3343) via *Agrobacterium tumefaciens* mediated transformation (Fillatti, *et al.*, *Bio/Technology* (1987) *5*:726-730) and regenerated. Preparation of pCGN3812, a tobacco SSU/SPS construct, and pCGN3815, a CaMV 35S/SPS construct are described in Examples 7.3 and 7.4, respectively. The fruit-specific E8/SPS constructs pCGN3342 and pCGN3343 were prepared as described for pCGN3812 with the following modifications. Approximately 2.1 kb of the 5' region corresponding to the tomato derived E8 fruit-specific promoter replace the SSU promoter region in pCGN3812. The E8 promoter is described in Deikmann *et al.* (1988) *EMBOJ*, 2:3315-3320; and Della Penna *et al.* (1989) *Plant Cell*, *1*:53-63. The pCGN3342 and pCGN3343 constructs also contain a SPS cDNA sequence truncated at the *Apo*I site just 3' of the SPS coding region (at nucleotide 3318), and fused to a 1.2 kb region of the *A. tumefaciens tml* 3' terminator region from pTiA6 (Barker *et al.*, (1983) *Plant Mol. Biol.*, 2:335-350; sequence 11208-10069 of the T-DNA region from *A. tumefaciens* Ti plasmid pTi15955). Constructs pCGN3342 and pCGN3343 represent opposite orientations of the E8-corn SPS-*tml* insert in the binary vector pCGN1557, which contains the kanamycin *npt*II marker gene under the control of the CaMV 35S promoter region and the *tml* 3' terminator region described above for pCGN3318 (McBride and Sumerfelt, *Plant Mol. Biol.* (1990) *14*:269-276). Tomato plant lines are designated with a number corresponding to the construct used for transformation. Tomato lines arising from separate transformation events are signified by a hyphen and a number following the construct/plant designation.

8.2 Immunoblot Results

Leaves from transformed tomato plants (pCGN3812 and pCGN3815) and control tomato and corn leaves were tested as described in Example 6.2 for SPS activity using the SPS #30 and SPS #90 peptide polyclonal antisera of Example 6. No cross reactivity between the antisera and the control (endogenous) tomato leaves was seen. This indicates

that the corn and tomato SPS are not highly related. As to the transgenic tomato plants, leaf extracts from tomato plants containing the pCGN3815 or pCGN3818 constructs showed signals up to levels several times those observed in the untransformed corn leaf extracts.

8.3 SPS Activity

Leaf extracts also were tested for SPS activity according to the resorcinol protocol described in Example 1.l.a. In comparison to leaf extracts from control plants, leaves from transformed tomato plants containing the SPS gene showed up to 12-fold increases in SPS activity. Higher SPS activity also was observed in some leaf extracts from transgenic tomato plants containing the corn SPS gene as compared to control corn leaf extracts.

8.4 Starch and Sucrose Levels

Leaf tissue was analyzed for starch and sucrose levels according to the method of Haissig, *et al.*, *Physiol. Plan* (1979) *47*:151-157. Two controls were used, leaves from an untransformed plant and leaves from a transformant which did not show any corn SPS immunoblot signal. The starch and sucrose levels of these two plants were essentially the same, and had an almost equal percentage of starch (mg/l00mg dry weight) and sucrose (mg/l0mg dry weight). High-expressing plants containing pCGN3812 (pCGN3812-9 and pCGN3812-11) showed both a reduction in leaf starch by 50% and an increase in sucrose levels by a factor of two. Thus, the extra sucrose synthesis provided by the exogenous SPS activity had a profound affect on carbohydrate partitioning. These data indicate that the presence of high levels of corn SPS activity resulting from a sufficient level of transgenic expression of a SPS transgene functional in tomato leaves cause a modification of carbohydrate partitioning in this tissue.

8.5 Oxygen Sensitivity

The interaction between photosynthesis and the synthesis of end products in tomatoes expressing corn SPS was evaluated by gas exchange analysis. Oxygen sensitivity of plants was induced by lowering growth temperature and then O_2 sensitivity measured as the rate of photosynthesis in low O_2 (Sage and Sharkey (1987) *Plant Physiol. 84*:658-664). Photosynthesis of tomato plants expressing corn SPS became oxygen insensitive at 14.2°C (measured in 35 Pa CO_2), whereas untransformed controls became insensitive at 17.3°C. Change in the growth temperature from 22°C to 30°C during the day did not

affect this pattern. Furthermore, the transformed plants did not acclimate following growth at high CO_2 (Worrell *et al.* (1991). *The Plant Cell* 3:1121-1131). These data show that the SPS expressing plants have a reduced ceiling imposed on photosynthesis by end product synthesis at lower temperatures. The data also show that the temperature at which photosynthesis becomes oxygen insensitive can be modulated by SPS activity through its effect on chloroplasts, photosynthetic capacity and end product synthesis and sink transport/conversion.

8.6 Temperature Effect on Partitioning

The effect of temperature on starch and sucrose partitioning was evaluated in tomato plants transformed with pCGN3812 (see 7.3). The transformed tomato plants were compared to control UC82B plants. The rate of starch plus sucrose synthesis as a function of temperature was assayed by feeding a pulse of $^{14}CO_2$ to leaves at a normal partial pressure then chasing with unlabeled CO_2 for a long enough period of time to permit incorporation of the labeled carbon into starch, sucrose, fructose, glucose or another end product but for a short enough period of time so that very little of the carbon was exported from the leaf source tissue. Analysis of end product synthesis showed that sucrose synthesis appeared more sensitive to temperature than did starch synthesis. For example, plants expressing about 5-fold more SPS activity compared to controls did not partition more carbon to sucrose at the lowest temperature. This indicates that the control coefficient for SPS approaches zero as metabolic activity of the plant is reduced with temperature under these conditions. The additional SPS activity also changed the oxygen sensitivity in this same temperature range. The above results show that partitioning between starch and sucrose, end-product synthesis/sink transport and conversion can be modulated as a function of temperature. (See Fig. 11).

8.7 Yield

Manipulation of yield by modification of end-product synthesis is related to growth conditions and reproductive/vegetative sink. The effect of growth conditions on tomato yield was evaluated in homozygous SSU/SPS (Rubisco small subunit promoter-SPS), 35/SPS (CaMV 355 promoter-SPS) and E8/SPS (E8 fruit-specific promoter-SPS) tomato plant lines grown under growth chamber, open-top chamber and field conditions following standard methods in the art.

When compared to untransformed tomato plants, variation in yield increase was

observed in the growth chamber, open-top chamber and field trials. Differences observed in fruit yield may be due to earlier flowering and the number of fruits set and filled for plants grown in growth chambers and pots compared to those grown in the field. Also, tomatoes expressing SPS behind the CaMV 35S promoter grew better than tomatoes expressing the gene behind a Rubisco small subunit promoter under growth chamber conditions. These data indicate a promoter effect. Additionally, studies in temperature controlled growth rooms show that there was more yield penalty in the SPS tomatoes at low temperatures than at high temperature. These data are in accordance with the partitioning data showing a reduction in modulation of sucrose levels at low temperature in tomato plants.

8.7.1 Soluble Solids In T2 SSU/SPS Tomato Plants Grown Under Growth Chamber and Greenhouse Conditions

Leaf-specific SSU/SPS tomato lines 3812-9 and 3812-11 were evaluated for soluble solid content. Extracts of fruit from these tomato lines and controls were grown and harvested in a Biotron growth chamber or under standard greenhouse conditions and served as the tissue source. T2 plants from the 3812-9 and 3812-11 lines were segregating as the original lines were shown to contain at least two SSU-SPS insertions. For growth chamber conditions, T2 plants were illuminated by metal halide lamps at peak level of 500 μmol photons/m/s (pot level), at a temperature of 26°C for the 16h day and 18°C at night, and a relative humidity of 60%. Plants were watered daily with half-strength Hoagland's solution (Hoagland and Arnon, *Calif. Argicult. Exp. Sta. Cir.* (1938) *357*:1-39). Soluble solids were evaluated as Brix units per unit weight fruit tissue measured for the average of three fruits per plant. Transgenic SSU/SPS plants grown under growth chamber conditions exhibited substantial increases in soluble solids compared to controls. The soluble solids measured in a segregating T2 population of 3812-11 plants grown under greenhouse conditions showed the same effect, but overall increases were reduced compared to SSU/SPS plants in growth chamber tests.

8.7.2 Soluble Solids In T4 SSU/SPS and 35S/SPS Tomato Plants Grown Under Greenhouse Conditions

Homozygous SSU/SPS tomato lines were generated from original SSU/SPS 3812-9 transformants in UC82-B tomatoes following standard products. Two homozygous lines

designated A and B were grown under greenhouse conditions and fruit evaluated for soluble solid content using Brix analysis measured per unit weight fruit tissue. Soluble solids were measured as an average of three plants per line and three fruit per plant. The average soluble solid content for the SSU/SPS 3812-9 lines was increased significantly compared to the UC82-B controls. The data was shown to be significant at a 0.01% level (99%), according to least significant difference (LSD) statistical analysis.

Homozygous lines of tomato plants transformed with the 35S/SPS construct of pCGN3815 were generated to compare the homozygous leaf-specific SPS construct results to homozygous constitutive expression construct. In one line, designated 3815-13-2, a substantial increase in fruit yield was observed, as measured for both fruit size and fruit number, compared to non-transformed controls and, surprisingly, compared against the SSU/SPS leaf-specific homozygous line controls. The 3815-13-2 plants also produced a second flush of fruit.

8.7.3 Soluble Solids In Field Grown T4 SSU/SPS Tomato Plants

Tomato plants homozygous for the SSU/SPS construct were generated from T4 crosses of original 3812-9 transformants as described in Example 8.1. Tomato lines designated A and B, which arose from separate crossing events, were grown under field conditions following standard field trial protocols. Soluble solids were obtained from fruit extracts of replicate plants as described for growth chamber and greenhouse studies. The soluble solids were evaluated by determining the average refractive index (RI) and specific sugar content per unit weight fruit tissue using high pressure liquid chromatography (HPLC). The RI measurements permitted analysis of overall sugar and acid content and the HPLC analysis for contributions by individual sugars. Both methods of analysis were conducted following standard protocols. The results are reported in Table 11 below.

Table 11

Soluble Solids and Sugar Content in Leaf-Specific SSU/SPS Tomato Plants

Tomato Line	RI	Sugar Concentration (%)			
		Sucrose	Glucose	Fructose	Total
Control	3.9	0.08	1.33	1.62	3.03
Control	4.2	0.11	1.51	1.75	3.37
(A) SSU/SPS-A-75-5	4.9	0.19	1.58	2.58	4.35
(A) SSU/SPS-A-91-4	4.9	0.19	1.61	2.55	4.35
(B) SSU/SPS-B-87-2	4.6	0.22	1.59	2.37	4.18
Average increase due to SPS	0.75	0.10	0.17	0.81	1.09

The transgenic tomato lines A and B consistently showed higher sugar and acid content compared to the controls. Sucrose, glucose and fructose levels were increased substantially in tomato fruit of the A and B lines, compared to the controls. Surprisingly, the contribution of glucose and fructose to the overall increase in soluble solids was pronounced compared to sucrose, indicating a net partitioning and conversion of photoassimilate to the fruit sink tissue.

8.7.4 Soluble Solids In Fruit-Specific E8/SPS Tomato Plants Grown Under Greenhouse Conditions

The soluble solids in fruit from tomato plant lines 3342 and 3343 expressing the fruit-specific E8-SPS constructs were evaluated as follows. Tomato plant lines arising from separate transformation events with pCGN3342 and pCGN3343 were grown under standard Greenhouse conditions. Soluble solids from replicate lines and trials were measured using RI, SPS specific activity and HLPC analyses. As a control, untransformed tomato plants and leaf-specific SSU/SPS tomato line were examined in parallel for each trial. Representative data for soluble solid content and distribution are reported in Tables 12-14 below.

Table 12

Soluble Solids In Fruit Specific E8/SPS Tomato Plants

Analysis	Transgenic Tomato Line (RI)[1]	Control Tomato Line (RI)[1]
	3343-6	UC82-B
A	7.2	6.0
B	8.2	5.2
C	10.2	7.5
	3342-11	UC82-B
D	7.9	4.9
E	7.6	6.2
	3343-22	UC82-B
F	7.8	6.8
G	8.5	7.0
H	7.6	6.6
I	8.2	
	3343-56	UC82-B
J	7.6	6.1
K	8.0	7.5
L	9.1	6.0

[1] Soluble solids measured as refractive index (RI) per unit weight fruit tissue.

Table 13

Soluble Solids and Sugar Content In Fruit-Specific E8/SPS Tomato Plants

Tomato Line	Date	RI	Sugar Concentration (%)			
			Sucrose	Glucose	Fructose	Total Sugars
Control	A	4.4	0.00	2.30	1.49	3.79
3342-11	B	7.9	0.00	3.64	3.29	6.93
3342-11	C	6.1	0.00	3.02	2.48	5.50
3342-11	D	7.5	0.00	3.12	3.27	6.39
3342-14	E	7.2	0.00	3.54	3.21	6.75
3342-14	F	8.4	0.00	4.13	3.72	7.85
3342-23	G	8.5	0.52	3.34	3.37	7.23
3343-5	H	4.0	0.00	1.66	1.27	2.93
3812-6	I	8.5	0.00	3.95	3.57	7.52

Table 14

Soluble Solids, Sugar Content and Acid Content In Fruit-Specific E8/SPS Tomato Plants

Tomato Line	Date	RI	Sugar Concentration (%)					Titratable Acidity
			Sucrose	Glucose	Fructose	Glucose/ Fructose	Total Sugars	
7060	A	6.6	N.D.	3.10	2.78	1.12	5.88	0.384
3343-22	B	8.2	N.D.	4.40	3.75	1.16	8.18	0.608
3342-16	C	8.2	N.D.	4.23	3.71	1.14	7.95	0.555
FL7060	D	6.2	N.D.	3.01	2.36	1.28	5.37	0.448
3343-56	E	8.1	N.D.	4.64	4.08	1.14	8.72	N.D.
3812-29	F	9.5	N.D.	5.13	4.21	1.22	9.33	N.D.
3343-6	G	10.4	0.36	4.91	4.69	1.05	9.97	.0597
3343-6	H	8.2	N.D.	3.95	3.68	1.07	7.63	.0640
FL7060	I	4.9	N.D.	2.33	1.79	1.30	4.11	0.432
FL7060	J	6.8	N.D.	3.46	2.84	1.21	6.29	0.533
3343-22	K	8.5	N.D.	4.24	3.57	1.19	7.81	N.D.

Tomato plant lines expressing the fruit-specific E8/SPS constructs consistently showed an increase in soluble solids reflected by overall sugar content, acid content and distribution. To assess the correlation between SPS activity and altered soluble solid content, SPS activity was measured in fruit from control tomato plants and compared to that in fruit from E8/SPS tomato lines 3343-6 and 3342-11. Control fruit from tomato line FL7060 was assayed with a SPS activity rate of 17.8 μmols sucrose/gram fresh weight/hour. Activity was much higher in the transgenic lines, with the 3343-6 event having a rate of 67.5 μmols sucrose/grown fresh weight/hour and the 3342-11 event measured at 36.6 μmols sucrose/gram fresh weight/hour. These results show that the increase of fruit-specific activity of the SPS correlates to the increase in sugar content of fruit.

Example 9

Transgenic SPS Potato Plants

9.1 Production of SPS Potato Plants

Potato plants were transformed with expression cassettes containing SPS coding sequences (pCGN3812) via *Agrobacterium tumefaciens* mediated transformation (Fillatti *et al.*, *supra*) and regenerated. Preparation of pCGN3812, a tobacco SSU/SPS construct, is described in Example 4.3.

9.2 Oxygen Sensitivity

Potato is adapted to cool weather and has a large vegetative sink, whereas the genetically similar tomato has a large reproductive sink. To evaluate whether potato has a relatively higher capacity for starch plus sucrose synthesis, allowing it to avoid oxygen insensitivity in the range of 12°C to 20°C, oxygen sensitivity was examined in potatoes expressing the corn SPS gene. Potatoes expressing the corn SPS exhibited a higher capacity for photosynthesis in elevated CO_2 when the plants were three weeks old compared to controls. When the potato corn SPS expressing plants were six to seven weeks old with developing tubers, they showed the acclimation to elevated CO_2 found in many plants and the controls (Fig. 12). These data show that responsiveness of plant growth to elevated CO_2 in plants having diverse physiological systems can be modulated by manipulating sucrose synthesis through an SPS which functions in plants.

9.3 Tuber Yield

Transformed potatoes expressing corn SPS exhibited greater tuber yield when grown in both large chambers and in open top chambers out-of-doors (Fig. 13). Because yield in potato is tuber mass and not fruit, the effect in potato appear different from the effect seen in tomato. Collectively, the tomato and potato yield data indicate that modification of SPS activity through expression of an exogenous transgene encoding SPS directly effects net sucrose synthesis and mass action in a similar manner in diverse plant systems, even though sucrose metabolism and its systemic effects may differ, which can be used to manipulate yield.

The above results demonstrate that transgenic plants can be constructed which have altered carbon partitioning through expression of a gene required for sucrose synthesis. Plants transformed with a DNA expression construct capable of controlling the expression of an SPS gene exhibited modification of starch and sucrose levels, CO_2 and/or O_2 sensitivity, temperature dependent growth responsiveness, and overall modification of carbon partitioning between source tissue such as leaf and sink tissue such as fruit or root. The data also show that the plant growth and yield were affected by altered carbon partitioning, as illustrated in two different plants of the nightshade family Solanaceae, potato and tomato. The data also show that control of carbohydrate partitioning through modification of end-product synthesis, for example, sucrose synthesis and conversion to other sugars in sink tissue, such as glucose and fructose provide means for altering plant growth and yield of specific plant tissues, plant parts and/or whole plant systems. In particular, increased SPS activity and tissue-specific SPS activity was demonstrated to produce a net increase in overall soluble solids in sink tissue such as fruit. Increases in the sugars sucrose, glucose and fructose represented soluble sugars analyzed in the soluble solids, with contributions by glucose and fructose being higher than sucrose. The SPS activity and sugar content data indicate that the endogenous acid invertase found in ripening tomato fruit contributed to the observed increases in glucose and fructose. Acid levels in the fruit-specific E8/SPS constructs also were observed, correlating acid content to an increase in sugar content. These data collectively show that SPS can be used to alter the overall content and ratio of soluble solids in a plant sink tissue, resulting in a demonstrable phenotype in plants, such as fruit having modified sweetness. Also, tomatoes expressing SPS behind the CaMV 35S promoter grew better than tomatoes expressing the gene behind a Rubisco small subunit promoter under growth chamber conditions. These data indicate a promoter effect which can be manipulated to control SPS activity in particular plant cells, plant

parts and throughout the plant. In general, the results show that plant growth and yield can be enhanced through transgenic expression of SPS, even though its effect on photosynthesis may be small.

Example 10

Soluble Solids in T2 SSU-SPS Plants

Investigation of the soluble solids in the fruits of the SSU-SPS lines was initially done on extracts from fruit of 3812-9 and 3812-11 lines grown in a Biotron incubator. T2 plants were illuminated by metal halide lamps at a peak level of 500 μmol photons/m/s (pot level), 26 C for the 16 h day and 18 C at night, and a relative humidity of 60%. Plants were watered daily with half-strength Hoagland's solution (Hoagland and Arnon, *Calif. Agricult. Exp. Sta. Cir.*(1938) *357*:1-39). These lines were segregating as the original lines contained at least 2 insertions.

Brix analysis (soluble solids) on extracts from these plants revealed lines with Brix readings as much as 40% higher than the controls. The extracts measured were the average of 3 fruit from one plant.

Measurements were also taken for fruit from a segregating T2 population of 3812-11 plants in the greenhouse. The controls averaged a Brix reading of 3.5 while the transgenics averaged 4.0, an increase of 14%.

Example 11

Homozygous Plants

T4 homozygous lines were generated from original 3812-9 transformants in UC82-B tomatoes. The original line segregated 15:1 for Kan resistance, indicating that it had two insertion sites. Two homozygous lines were generated and verified to be different by Southern border analysis. These lines were designated A and B.

Individual homozygote (T4) lines were grown in the greenhouse, with three fruit taken from each plant and 3 plants analyzed from each line. The Brix of the UC82B controls was 3.35 while the Brix on the 3812-9 lines ranged from 3.7 to 4.1. This is an increase from 12% to 24%. Statistics (LSD) on all the lines in which fruit from 3 plants were analyzed showed these results to be significant at a .01% level (99%).

Measurements were also made on homozygous lines of tomato plants transformed with the 35S CaMV promoter-SPS construct pCGN3815. In one line, 3815-13-2 there was a substantial increase in yield of tomatoes, in terms of an increase in both fruit size and in fruit number, as measured against non-transformed control plants and as against SSU-SPS homozygous line controls. The 3815-13-2 plants also produced a second flush of fruit. A second transgenic line containing the pCGN3815 construct did not produce these dramatic yield increases.

Example 12

Brix Analysis of Field Trial SSU-SPS Fruit

Field trial results of RI measurements are provided in Table 15. The R/I (refractive index) was measured several times on the fruit of these plants (Table 15). R/I is a measure of soluble solids and is indicative of sugars and acids. The transgenic A and B lines consistently had a higher R/I than the control UC82-B.

Table 15

Summary of Refractive Index Measurements

Line	Reading 1	Reading 2	Reading 3	Reading 4	Overall Avg
Trial 1					
UC82-B	3.3	3.9	3.6	3.0	3.5
A Lines (X2)	4.0	4.4	4.6	3.8	4.2
Trial 2					
UC82-B	4.1	4.1	3.9		4.0
A Lines (X3)	4.8	4.3	4.2		4.4
B Lines (X2)	4.4	4.4	4.2		4.3
Trial 3					
UC82B	3.2				
A Lines (X1)	4.2				
B Lines (X1)					

Example 13

HPLC Analysis on SSU-SPS Fruit Sugars

Fruit from the SPS plants described in Example 12 were further analyzed by HPLC to determine contributions of individual sugars to the increased soluble solids content. As seen in Table 16, sucrose did not increase as much as might be expected based on the fact that sucrose is the sugar transported by the plant into the fruit. Glucose was not increased as much as fructose, which increased nearly 50%.

It is evident from the above results, that plant cells and plants can be produced which have improved properties or may produce a desired phenotype. In accordance with the subject invention, it is now seen that SPS sequences may be introduced into a plant host cell and be used to express the enzyme to increase soluble solids content in fruit. Moreover, it is seen that the SPS may be used to alter the overall content and ratio of soluble solids in plant sink tissue, resulting in a demonstrable phenotype *in planta*, such as altered fruit sweetness. In this manner, fruits, such as tomato fruit, having modified sweetness may be obtained.

Example 14

Fruit Specific Expression of SPS

E8-SPS constructs designated as pCGN3342 and pCGN3343 contain the tomato E8 promoter comprising the approximately 2.1 kb 5' region of the E8 promoter. A description of this promoter region can be found in Deikman *et al.*, *supra*, and in Deikman *et al.* (*Plant Physiol.* (1992) *100*:2013-2017).

This E8 promoter is fused to the same SPS encoding sequence used for pCGN3812 and pCGN3815, only the SPS sequence used in these constructs has been truncated at the ApoI site just 3' of the SPS encoding sequence (at nucleotide 3318), and fused to a 1.2 kb region of the *tml* 3' region from pTiA6 (Barker *et al.*, (1983) *Plant Mol. Biol. 2*:335-350; sequence 11207-10069 of the T-DNA region from the *Agrobacterium tumefaciens* Ti plasmid pTi15955). Constructs pCGN3342 and pCGN3343 are the opposite orientations of this E8-SPS-*tml* construct in the 35S kan binary, pCGN1557 (McBride and Summerfelt, *supra*). Tomato lines arising from separate transformation events using pCGN3342 and pCGN3343 are signified by the construct number followed by a hyphen and an event number.

Table 17 provides data from RI measurements of soluble solids in tomatoes from

greenhouse studies of T1 plants. The RI was measured several times on the fruit of these plants.

Assays were made for the SPS activity in control and transgenic fruit from the 3343-6 and 3342-11 events. The control 7060 fruit was assayed with a SPS activity rate of 17.8 μmols sucrose/g/hr. This demonstrates that the increase sugar concentration of fruit in transgenic tomatoes over the control correlates to an increase of SPS activity in the fruit.

Tables 18 and 19 provide an analysis of individual sugars as measured by HPLC from two separate trials, to determine contributions of each sugar to the increased soluble solids content observed in transgenic E8-SPS fruit. The data of Table 18 and 19 demonstrate that increased SPS activity from transgenic expression in fruit by a fruit specific promoter can produce an overall net increase in sugars in the fruit. Due to the endogenous acid invertase found in ripening tomato fruit, increases in sugar are found in glucose and fructose.

It also appears that there is a correlating increase in acid levels with an increase in sugar content in fruit transformed with E8-SPS.

Table 16

Sugars of Tomatoes of Plants Transformed with SPS Gene

			Sugar Concentration (%)				
ID#	Tomato Line	RI	Sucrose	Glucose	Fructose	Total	Relative Increase Over Control
41000 #1	Control	3.9	0.08	1.33	1.62	3.03	
41000 #2	Control	4.2	0.11	1.51	1.75	3.37	
41003	SSU-SPS-A-75-5	4.9	0.19	1.58	2.58	4.35	36%
41004	SSU-SPS-A-91-4	4.9	0.19	1.61	2.55	4.35	36%
41008	SSU-SPS-B-87-2	4.6	0.22	1.59	2.37	4.18	31%
Average increase due to SPS		0.18	0.10	0.17	0.81	1.09	34%

Table 17

	Date	RI of Transgenic	RI of Control
3343-6	A	7.2	6.0
	B	8.2	5.2
	C	10.2	7.5
3342-11	D	7.9	4.9
		7.6	6.2
3343-22	E	7.8	6.8
	F	8.5	6.8
	G	7.6	7.0
	H	8.2	6.6
3343-56	I	7.6	6.1
	J	8.0	7.5
	K	9.1	6.0

Table 18

Sugars of SPS Tomato Lines

Line ID	Date	RI	Sugar Content (%)			
			Sucrose	Glucose	Fructose	Total
Control	A	4.4	0.00	2.30	1.49	3.79
3342-11	B	7.9	0.00	3.64	3.29	6.93
3342-11	C	6.1	0.00	3.02	2.48	5.50
3342-11	D	7.5	0.00	3.12	3.27	6.39
3342-14	E	7.2	0.00	3.54	3.21	6.75
3342-14	F	8.4	0.00	4.13	3.72	7.85
3342-23	G	8.5	0.52	3.34	3.37	7.23
3343-5	H	4.0	0.00	1.66	1.27	2.93
3812-6	I	8.5	0.00	3.95	3.57	7.52

Table 19

Sugars and Acids of SPS Tomato Lines

Line ID	Date	RI	Sucrose	Glucose	Fructose	G/F	Total Sugars	Titratable Acidity
7060	A	6.6	N.D.	3.10	2.78	1.12	5.88	0.384
3343-22	B	8.2	N.D.	4.40	3.79	1.16	8.18	0.608
3342-16	C	8.2	N.D.	4.23	3.71	1.14	7.94	0.555
FL7060	D	6.2	N.D.	3.01	2.36	1.28	5.37	0.448
3343-56	E	8.1	N.D.	4.64	4.08	1.14	8.72	--
3812-29	F	9.5	N.D.	5.13	4.21	1.22	9.33	--
3343-6	G	10.4	.36	4.91	4.69	1.05	9.97	.0597
3343-6	H	8.2	N.D.	3.95	3.68	1.07	7.63	.0640
FL7060	I	4.9	N.D.	2.33	1.79	1.30	4.11	0.432
FL7060	J	6.8	N.D.	3.46	2.84	1.21	6.29	0.533
3343-22	K	8.5	N.D.	4.24	3.57	1.19	7.81	--

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All referenced publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now been fully described, it would be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Van Assche, C.
Lando, D.
Bruneau, J. M.
Voelker, T.
Gervais, M.

(ii) TITLE OF INVENTION: MODIFICATION OF SUCROSE PHOSPHATE SYNTHASE IN PLANTS

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Law Offices of Barbara Rae-Venter
(B) STREET: 260 Sheridan Avenue, Suite 440
(C) CITY: Palo Alto
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94306

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: NOT YET ASSIGNED
(B) FILING DATE: NOT YET ASSIGNED
(C) CLASSIFICATION: NOT YET ASSIGNED

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/175,471
(B) FILING DATE: 27-DEC-1993

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Barbara Rae-Venter
(B) REGISTRATION NUMBER: 32,750
(C) REFERENCE/DOCKET NUMBER: CGNE.072.02US

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415)328-4400
(B) TELEFAX: (415)328-4477

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Thr Trp Ile Lys
1

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr Val Val Glu Leu Ala Arg
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Met Pro Pro Ile Trp Ala Glu Val Met Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Leu Arg Pro Asp Gln Asp Tyr Leu Met His Ile Ser His Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Trp Ser His Asp Gly Ala Arg
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3509 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 112..3315

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
GAATTCCGGC GTGGGCGCTG GCTAGTGCT CCCGCAGCGA GCGATCTGAG AGAACGGTAG      60

AGTTCCGGCC GGGCGCGCGG GAGAGGAGGA GGGTCGGGCG GGGAGGATCC G ATG GCC     117
                                                          Met Ala
                                                            1

GGG AAC GAG TGG ATC AAT GGG TAC CTG GAG GCG ATC CTC GAC AGC CAC      165
Gly Asn Glu Trp Ile Asn Gly Tyr Leu Glu Ala Ile Leu Asp Ser His
          5                  10                  15

ACC TCG TCG CGG GGT GCC GGC GGC GGC GGC GGC GGG GGG GAC CCC AGG      213
Thr Ser Ser Arg Gly Ala Gly Gly Gly Gly Gly Gly Gly Asp Pro Arg
     20                  25                  30

TCG CCG ACG AAG GCG GCG AGC CCC CGC GGC GCG CAC ATG AAC TTC AAC      261
Ser Pro Thr Lys Ala Ala Ser Pro Arg Gly Ala His Met Asn Phe Asn
 35                  40                  45                  50

CCC TCG CAC TAC TTC GTC GAG GAG GTG GTC AAG GGC GTC GAC GAG AGC      309
Pro Ser His Tyr Phe Val Glu Glu Val Val Lys Gly Val Asp Glu Ser
                 55                  60                  65

GAC CTC CAC CGG ACG TGG ATC AAG GTC GTC GCC ACC CGC AAC GCC CGC      357
Asp Leu His Arg Thr Trp Ile Lys Val Val Ala Thr Arg Asn Ala Arg
             70                  75                  80
```

```
GAG CGC AGC ACC AGG CTC GAG AAC ATG TGC TGG CGG ATC TGG CAC CTC      405
Glu Arg Ser Thr Arg Leu Glu Asn Met Cys Trp Arg Ile Trp His Leu
         85                  90                  95

GCG CGC AAG AAG AAG CAG CTG GAG CTG GAG GGC ATC CAG AGA ATC TCG      453
Ala Arg Lys Lys Lys Gln Leu Glu Leu Glu Gly Ile Gln Arg Ile Ser
    100                 105                 110

GCA AGA AGG AAG GAA CAG GAG CAG GTG CGT CGT GAG GCG ACG GAG GAC      501
Ala Arg Arg Lys Glu Gln Glu Gln Val Arg Arg Glu Ala Thr Glu Asp
115                 120                 125                 130

CTG GCC GAG GAT CTG TCA GAA GGC GAG AAG GGA GAC ACC ATC GGC GAG      549
Leu Ala Glu Asp Leu Ser Glu Gly Glu Lys Gly Asp Thr Ile Gly Glu
                135                 140                 145

CTT GCG CCG GTT GAG ACG ACC AAG AAG AAG TTC CAG AGG AAC TTC TCT      597
Leu Ala Pro Val Glu Thr Thr Lys Lys Lys Phe Gln Arg Asn Phe Ser
            150                 155                 160

GAC CTT ACC GTC TGG TCT GAC GAC AAT AAG GAG AAG AAG CTT TAC ATT      645
Asp Leu Thr Val Trp Ser Asp Asp Asn Lys Glu Lys Lys Leu Tyr Ile
        165                 170                 175

GTG CTC ATC AGC GTG CAT GGT CTT GTT CGT GGA GAA AAC ATG GAA CTA      693
Val Leu Ile Ser Val His Gly Leu Val Arg Gly Glu Asn Met Glu Leu
    180                 185                 190

GGT CGT GAT TCT GAT ACA GGT GGC CAG GTG AAA TAT GTG GTC GAA CTT      741
Gly Arg Asp Ser Asp Thr Gly Gly Gln Val Lys Tyr Val Val Glu Leu
195                 200                 205                 210

GCA AGA GCG ATG TCA ATG ATG CCT GGA GTG TAC AGG GTG GAC CTC TTC      789
Ala Arg Ala Met Ser Met Met Pro Gly Val Tyr Arg Val Asp Leu Phe
                215                 220                 225

ACT CGT CAA GTG TCA TCT CCT GAC GTG GAC TGG AGC TAC GGT GAG CCA      837
Thr Arg Gln Val Ser Ser Pro Asp Val Asp Trp Ser Tyr Gly Glu Pro
            230                 235                 240

ACC GAG ATG TTA TGC GCC GGT TCC AAT GAT GGA GAG GGG ATG GGT GAG      885
Thr Glu Met Leu Cys Ala Gly Ser Asn Asp Gly Glu Gly Met Gly Glu
        245                 250                 255
```

```
AGT GGC GGA GCC TAC ATT GTG CGC ATA CCG TGT GGG CCG CGG GAT AAA      933
Ser Gly Gly Ala Tyr Ile Val Arg Ile Pro Cys Gly Pro Arg Asp Lys
    260                 265                 270

TAC CTC AAG AAG GAA GCG TTG TGG CCT TAC CTC CAA GAG TTT GTC GAT      981
Tyr Leu Lys Lys Glu Ala Leu Trp Pro Tyr Leu Gln Glu Phe Val Asp
275                 280                 285                 290

GGA GCC CTT GCG CAT ATC CTG AAC ATG TCC AAG GCT CTG GGA GAG CAG     1029
Gly Ala Leu Ala His Ile Leu Asn Met Ser Lys Ala Leu Gly Glu Gln
                295                 300                 305

GTT GGA AAT GGG AGG CCA GTA CTG CCT TAC GTG ATA CAT GGG CAC TAT     1077
Val Gly Asn Gly Arg Pro Val Leu Pro Tyr Val Ile His Gly His Tyr
            310                 315                 320

GCC GAT GCT GGA GAT GTT GCT GCT CTC CTT TCT GGT GCG CTG AAT GTG     1125
Ala Asp Ala Gly Asp Val Ala Ala Leu Leu Ser Gly Ala Leu Asn Val
        325                 330                 335

CCA ATG GTG CTC ACT GGC CAC TCA CTT GGG AGG AAC AAG CTG GAA CAA     1173
Pro Met Val Leu Thr Gly His Ser Leu Gly Arg Asn Lys Leu Glu Gln
    340                 345                 350

CTG CTG AAG CAA GGG CGC ATG TCC AAG GAG GAG ATC GAT TCG ACA TAC     1221
Leu Leu Lys Gln Gly Arg Met Ser Lys Glu Glu Ile Asp Ser Thr Tyr
355                 360                 365                 370

AAG ATC ATG AGG CGT ATC GAG GGT GAG GAG CTG GCC CTG GAT GCG TCA     1269
Lys Ile Met Arg Arg Ile Glu Gly Glu Glu Leu Ala Leu Asp Ala Ser
                375                 380                 385

GAG CTT GTA ATC ACG AGC ACA AGG CAG GAG ATT GAT GAG CAG TGG GGA     1317
Glu Leu Val Ile Thr Ser Thr Arg Gln Glu Ile Asp Glu Gln Trp Gly
            390                 395                 400

TTG TAC GAT GGA TTT GAT GTC AAG CTT GAG AAA GTG CTG AGG GCA CGG     1365
Leu Tyr Asp Gly Phe Asp Val Lys Leu Glu Lys Val Leu Arg Ala Arg
        405                 410                 415

GCG AGG CGC GGG GTT AGC TGC CAT GGT CGT TAC ATG CCT AGG ATG GTG     1413
Ala Arg Arg Gly Val Ser Cys His Gly Arg Tyr Met Pro Arg Met Val
    420                 425                 430
```

```
GTG ATT CCT CCG GGA ATG GAT TTC AGC AAT GTT GTA GTT CAT GAA GAC      1461
Val Ile Pro Pro Gly Met Asp Phe Ser Asn Val Val Val His Glu Asp
435                 440                 445                 450

ATT GAT GGG GAT GGT GAC GTC AAA GAT GAT ATC GTT GGT TTG GAG GGT      1509
Ile Asp Gly Asp Gly Asp Val Lys Asp Asp Ile Val Gly Leu Glu Gly
                455                 460                 465

GCC TCA CCC AAG TCA ATG CCC CCA ATT TGG GCC GAA GTG ATG CGG TTC      1557
Ala Ser Pro Lys Ser Met Pro Pro Ile Trp Ala Glu Val Met Arg Phe
            470                 475                 480

CTG ACC AAC CCT CAC AAG CCG ATG ATC CTG GCG TTA TCA AGA CCA GAC      1605
Leu Thr Asn Pro His Lys Pro Met Ile Leu Ala Leu Ser Arg Pro Asp
        485                 490                 495

CCG AAG AAG AAC ATC ACT ACC CTC GTC AAA GCC TTT GGA GAG TGT CGT      1653
Pro Lys Lys Asn Ile Thr Thr Leu Val Lys Ala Phe Gly Glu Cys Arg
    500                 505                 510

CCA CTC AGG GAA CTT GCA AAC CTT ACT CTG ATC ATG GGT AAC AGA GAT      1701
Pro Leu Arg Glu Leu Ala Asn Leu Thr Leu Ile Met Gly Asn Arg Asp
515                 520                 525                 530

GAC ATC GAC GAC ATG TCT GCT GGC AAT GCC AGT GTC CTC ACC ACA GTT      1749
Asp Ile Asp Asp Met Ser Ala Gly Asn Ala Ser Val Leu Thr Thr Val
                535                 540                 545

CTG AAG CTG ATT GAC AAG TAT GAT CTG TAC GGA AGC GTG GCG TTC CCT      1797
Leu Lys Leu Ile Asp Lys Tyr Asp Leu Tyr Gly Ser Val Ala Phe Pro
            550                 555                 560

AAG CAT CAC AAT CAG GCT GAC GTC CCG GAG ATC TAT CGC CTC GCG GCC      1845
Lys His His Asn Gln Ala Asp Val Pro Glu Ile Tyr Arg Leu Ala Ala
        565                 570                 575

AAA ATG AAG GGC GTC TTC ATC AAC CCT GCT CTC GTT GAG CCG TTT GGT      1893
Lys Met Lys Gly Val Phe Ile Asn Pro Ala Leu Val Glu Pro Phe Gly
    580                 585                 590

CTC ACC CTG ATC GAG GCT GCG GCA CAC GGA CTC CCG ATA GTC GCT ACC      1941
Leu Thr Leu Ile Glu Ala Ala Ala His Gly Leu Pro Ile Val Ala Thr
595                 600                 605                 610
```

```
AAG AAT GGT GGT CCG GTC GAC ATT ACA AAT GCA TTA AAC AAC GGA CTG      1989
Lys Asn Gly Gly Pro Val Asp Ile Thr Asn Ala Leu Asn Asn Gly Leu
                615                 620                 625

CTC GTT GAC CCA CAC GAC CAG AAC GCC ATC GCT GAT GCA CTG CTG AAG      2037
Leu Val Asp Pro His Asp Gln Asn Ala Ile Ala Asp Ala Leu Leu Lys
            630                 635                 640

CTT GTG GCA GAC AAG AAC CTG TGG CAG GAA TGC CGG AGA AAC GGG CTG      2085
Leu Val Ala Asp Lys Asn Leu Trp Gln Glu Cys Arg Arg Asn Gly Leu
        645                 650                 655

CGC AAC ATC CAC CTC TAC TCA TGG CCG GAG CAC TGC CGC ACT TAC CTC      2133
Arg Asn Ile His Leu Tyr Ser Trp Pro Glu His Cys Arg Thr Tyr Leu
    660                 665                 670

ACC AGG GTG GCC GGG TGC CGG TTA AGG AAC CCG AGG TGG CTG AAG GAC      2181
Thr Arg Val Ala Gly Cys Arg Leu Arg Asn Pro Arg Trp Leu Lys Asp
675                 680                 685                 690

ACA CCA GCA GAT GCC GGA GCC GAT GAG GAG GAG TTC CTG GAG GAT TCC      2229
Thr Pro Ala Asp Ala Gly Ala Asp Glu Glu Glu Phe Leu Glu Asp Ser
                695                 700                 705

ATG GAC GCT CAG GAC CTG TCA CTC CGT CTG TCC ATC GAC GGT GAG AAG      2277
Met Asp Ala Gln Asp Leu Ser Leu Arg Leu Ser Ile Asp Gly Glu Lys
            710                 715                 720

AGC TCG CTG AAC ACT AAC GAT CCA CTG TGG TTC GAC CCC CAG GAT CAA      2325
Ser Ser Leu Asn Thr Asn Asp Pro Leu Trp Phe Asp Pro Gln Asp Gln
        725                 730                 735

GTG CAG AAG ATC ATG AAC AAC ATC AAG CAG TCG TCA GCG CTT CCT CCG      2373
Val Gln Lys Ile Met Asn Asn Ile Lys Gln Ser Ser Ala Leu Pro Pro
    740                 745                 750

TCC ATG TCC TCA GTC GCA GCC GAG GGC ACA GGC AGC ACC ATG AAC AAA      2421
Ser Met Ser Ser Val Ala Ala Glu Gly Thr Gly Ser Thr Met Asn Lys
755                 760                 765                 770

TAC CCA CTC CTG CGC CGG CGC CGG CGC TTG TTC GTC ATA GCT GTG GAC      2469
Tyr Pro Leu Leu Arg Arg Arg Arg Arg Leu Phe Val Ile Ala Val Asp
                775                 780                 785
```

```
TGC TAC CAG GAC GAT GGC CGT GCT AGC AAG AAG ATG CTG CAG GTG ATC      2517
Cys Tyr Gln Asp Asp Gly Arg Ala Ser Lys Lys Met Leu Gln Val Ile
            790                 795                 800

CAG GAA GTT TTC AGA GCA GTC CGA TCG GAC TCC CAG ATG TTC AAG ATC      2565
Gln Glu Val Phe Arg Ala Val Arg Ser Asp Ser Gln Met Phe Lys Ile
        805                 810                 815

TCA GGG TTC ACG CTG TCG ACT GCC ATG CCG TTG TCC GAG ACA CTC CAG      2613
Ser Gly Phe Thr Leu Ser Thr Ala Met Pro Leu Ser Glu Thr Leu Gln
    820                 825                 830

CTT CTG CAG CTC GGC AAG ATC CCA GCG ACC GAC TTC GAC GCC CTC ATC      2661
Leu Leu Gln Leu Gly Lys Ile Pro Ala Thr Asp Phe Asp Ala Leu Ile
835                 840                 845                 850

TGT GGC AGC GGC AGC GAG GTG TAC TAT CCT GGC ACG GCG AAC TGC ATG      2709
Cys Gly Ser Gly Ser Glu Val Tyr Tyr Pro Gly Thr Ala Asn Cys Met
                855                 860                 865

GAC GCT GAA GGA AAG CTG CGC CCA GAT CAG GAC TAT CTG ATG CAC ATC      2757
Asp Ala Glu Gly Lys Leu Arg Pro Asp Gln Asp Tyr Leu Met His Ile
            870                 875                 880

AGC CAC CGC TGG TCC CAT GAC GGC GCG AGG CAG ACC ATA GCG AAG CTC      2805
Ser His Arg Trp Ser His Asp Gly Ala Arg Gln Thr Ile Ala Lys Leu
        885                 890                 895

ATG GGC GCT CAG GAC GGT TCA GGC GAC GCT GTC GAG CAG GAC GTG GCG      2853
Met Gly Ala Gln Asp Gly Ser Gly Asp Ala Val Glu Gln Asp Val Ala
    900                 905                 910

TCC AGT AAT GCA CAC TGT GTC GCG TTC CTC ATC AAA GAC CCC CAA AAG      2901
Ser Ser Asn Ala His Cys Val Ala Phe Leu Ile Lys Asp Pro Gln Lys
915                 920                 925                 930

GTG AAA ACG GTC GAT GAG ATG AGG GAG CGG CTG AGG ATG CGT GGT CTC      2949
Val Lys Thr Val Asp Glu Met Arg Glu Arg Leu Arg Met Arg Gly Leu
                935                 940                 945

CGC TGC CAC ATC ATG TAC TGC AGG AAC TCG ACA AGG CTT CAG GTT GTC      2997
Arg Cys His Ile Met Tyr Cys Arg Asn Ser Thr Arg Leu Gln Val Val
            950                 955                 960
```

```
CCT CTG CTA GCA TCA AGG TCA CAG GCA CTC AGG TAT CTT TCC GTG CGC      3045
Pro Leu Leu Ala Ser Arg Ser Gln Ala Leu Arg Tyr Leu Ser Val Arg
        965                 970                 975

TGG GGC GTA TCT GTG GGG AAC ATG TAT CTG ATC ACC GGG GAA CAT GGC      3093
Trp Gly Val Ser Val Gly Asn Met Tyr Leu Ile Thr Gly Glu His Gly
    980                 985                 990

GAC ACC GAT CTA GAG GAG ATG CTA TCC GGG CTA CAC AAG ACC GTG ATC      3141
Asp Thr Asp Leu Glu Glu Met Leu Ser Gly Leu His Lys Thr Val Ile
995                1000                1005                1010

GTC CGT GGC GTC ACC GAG AAG GGT TCG GAA GCA CTG GTG AGG AGC CCA      3189
Val Arg Gly Val Thr Glu Lys Gly Ser Glu Ala Leu Val Arg Ser Pro
                1015                1020                1025

GGA AGC TAC AAG AGG GAC GAT GTC GTC CCG TCT GAG ACC CCC TTG GCT      3237
Gly Ser Tyr Lys Arg Asp Asp Val Val Pro Ser Glu Thr Pro Leu Ala
            1030                1035                1040

GCG TAC ACG ACT GGT GAG CTG AAG GCC GAC GAG ATC ATG CGG GCT CTG      3285
Ala Tyr Thr Thr Gly Glu Leu Lys Ala Asp Glu Ile Met Arg Ala Leu
        1045                1050                1055

AAG CAA GTC TCC AAG ACT TCC AGC GGC ATG TGAATTTGAT GCTTCTTTTA        3335
Lys Gln Val Ser Lys Thr Ser Ser Gly Met
    1060                1065

CATTTTGTCC TTTTCTTCAC TGCTATATAA AATAAGTTGT GAACAGTACC GCGGGTGTGT    3395

ATATATATAT TGCAGTGACA AATAAAACAG GACACTGCTA ACTATACTGG TGAATATACG    3455

ACTGTCAAGA TTGTATGCTA AGTACTCCAT TTCTCAATGT ATCAATCGGA ATTC          3509
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1068 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Ala Gly Asn Glu Trp Ile Asn Gly Tyr Leu Glu Ala Ile Leu Asp
 1               5                  10                  15

Ser His Thr Ser Ser Arg Gly Ala Gly Gly Gly Gly Gly Gly Gly Asp
            20                  25                  30

Pro Arg Ser Pro Thr Lys Ala Ala Ser Pro Arg Gly Ala His Met Asn
        35                  40                  45

Phe Asn Pro Ser His Tyr Phe Val Glu Glu Val Val Lys Gly Val Asp
    50                  55                  60

Glu Ser Asp Leu His Arg Thr Trp Ile Lys Val Val Ala Thr Arg Asn
65                  70                  75                  80

Ala Arg Glu Arg Ser Thr Arg Leu Glu Asn Met Cys Trp Arg Ile Trp
                85                  90                  95

His Leu Ala Arg Lys Lys Lys Gln Leu Glu Leu Glu Gly Ile Gln Arg
            100                 105                 110

Ile Ser Ala Arg Arg Lys Glu Gln Glu Gln Val Arg Arg Glu Ala Thr
        115                 120                 125

Glu Asp Leu Ala Glu Asp Leu Ser Glu Gly Glu Lys Gly Asp Thr Ile
    130                 135                 140

Gly Glu Leu Ala Pro Val Glu Thr Thr Lys Lys Lys Phe Gln Arg Asn
145                 150                 155                 160

Phe Ser Asp Leu Thr Val Trp Ser Asp Asp Asn Lys Glu Lys Lys Leu
                165                 170                 175

Tyr Ile Val Leu Ile Ser Val His Gly Leu Val Arg Gly Glu Asn Met
            180                 185                 190

Glu Leu Gly Arg Asp Ser Asp Thr Gly Gly Gln Val Lys Tyr Val Val
        195                 200                 205

Glu Leu Ala Arg Ala Met Ser Met Met Pro Gly Val Tyr Arg Val Asp
    210                 215                 220
```

```
Leu Phe Thr Arg Gln Val Ser Ser Pro Asp Val Asp Trp Ser Tyr Gly
225                 230                 235                 240

Glu Pro Thr Glu Met Leu Cys Ala Gly Ser Asn Asp Gly Glu Gly Met
                245                 250                 255

Gly Glu Ser Gly Gly Ala Tyr Ile Val Arg Ile Pro Cys Gly Pro Arg
            260                 265                 270

Asp Lys Tyr Leu Lys Lys Glu Ala Leu Trp Pro Tyr Leu Gln Glu Phe
        275                 280                 285

Val Asp Gly Ala Leu Ala His Ile Leu Asn Met Ser Lys Ala Leu Gly
    290                 295                 300

Glu Gln Val Gly Asn Gly Arg Pro Val Leu Pro Tyr Val Ile His Gly
305                 310                 315                 320

His Tyr Ala Asp Ala Gly Asp Val Ala Ala Leu Leu Ser Gly Ala Leu
                325                 330                 335

Asn Val Pro Met Val Leu Thr Gly His Ser Leu Gly Arg Asn Lys Leu
            340                 345                 350

Glu Gln Leu Leu Lys Gln Gly Arg Met Ser Lys Glu Glu Ile Asp Ser
        355                 360                 365

Thr Tyr Lys Ile Met Arg Arg Ile Glu Gly Glu Glu Leu Ala Leu Asp
    370                 375                 380

Ala Ser Glu Leu Val Ile Thr Ser Thr Arg Gln Glu Ile Asp Glu Gln
385                 390                 395                 400

Trp Gly Leu Tyr Asp Gly Phe Asp Val Lys Leu Glu Lys Val Leu Arg
                405                 410                 415

Ala Arg Ala Arg Arg Gly Val Ser Cys His Gly Arg Tyr Met Pro Arg
            420                 425                 430

Met Val Val Ile Pro Pro Gly Met Asp Phe Ser Asn Val Val Val His
        435                 440                 445

Glu Asp Ile Asp Gly Asp Gly Asp Val Lys Asp Asp Ile Val Gly Leu
    450                 455                 460
```

```
Glu Gly Ala Ser Pro Lys Ser Met Pro Pro Ile Trp Ala Glu Val Met
465                 470                 475                 480

Arg Phe Leu Thr Asn Pro His Lys Pro Met Ile Leu Ala Leu Ser Arg
                485                 490                 495

Pro Asp Pro Lys Lys Asn Ile Thr Thr Leu Val Lys Ala Phe Gly Glu
            500                 505                 510

Cys Arg Pro Leu Arg Glu Leu Ala Asn Leu Thr Leu Ile Met Gly Asn
        515                 520                 525

Arg Asp Asp Ile Asp Asp Met Ser Ala Gly Asn Ala Ser Val Leu Thr
    530                 535                 540

Thr Val Leu Lys Leu Ile Asp Lys Tyr Asp Leu Tyr Gly Ser Val Ala
545                 550                 555                 560

Phe Pro Lys His His Asn Gln Ala Asp Val Pro Glu Ile Tyr Arg Leu
                565                 570                 575

Ala Ala Lys Met Lys Gly Val Phe Ile Asn Pro Ala Leu Val Glu Pro
            580                 585                 590

Phe Gly Leu Thr Leu Ile Glu Ala Ala Ala His Gly Leu Pro Ile Val
        595                 600                 605

Ala Thr Lys Asn Gly Gly Pro Val Asp Ile Thr Asn Ala Leu Asn Asn
    610                 615                 620

Gly Leu Leu Val Asp Pro His Asp Gln Asn Ala Ile Ala Asp Ala Leu
625                 630                 635                 640

Leu Lys Leu Val Ala Asp Lys Asn Leu Trp Gln Glu Cys Arg Arg Asn
                645                 650                 655

Gly Leu Arg Asn Ile His Leu Tyr Ser Trp Pro Glu His Cys Arg Thr
            660                 665                 670

Tyr Leu Thr Arg Val Ala Gly Cys Arg Leu Arg Asn Pro Arg Trp Leu
        675                 680                 685

Lys Asp Thr Pro Ala Asp Ala Gly Ala Asp Glu Glu Glu Phe Leu Glu
    690                 695                 700
```

```
Asp Ser Met Asp Ala Gln Asp Leu Ser Leu Arg Leu Ser Ile Asp Gly
705                 710                 715                 720

Glu Lys Ser Ser Leu Asn Thr Asn Asp Pro Leu Trp Phe Asp Pro Gln
                725                 730                 735

Asp Gln Val Gln Lys Ile Met Asn Asn Ile Lys Gln Ser Ser Ala Leu
            740                 745                 750

Pro Pro Ser Met Ser Ser Val Ala Ala Glu Gly Thr Gly Ser Thr Met
        755                 760                 765

Asn Lys Tyr Pro Leu Leu Arg Arg Arg Arg Arg Leu Phe Val Ile Ala
    770                 775                 780

Val Asp Cys Tyr Gln Asp Asp Gly Arg Ala Ser Lys Lys Met Leu Gln
785                 790                 795                 800

Val Ile Gln Glu Val Phe Arg Ala Val Arg Ser Asp Ser Gln Met Phe
                805                 810                 815

Lys Ile Ser Gly Phe Thr Leu Ser Thr Ala Met Pro Leu Ser Glu Thr
            820                 825                 830

Leu Gln Leu Leu Gln Leu Gly Lys Ile Pro Ala Thr Asp Phe Asp Ala
        835                 840                 845

Leu Ile Cys Gly Ser Gly Ser Glu Val Tyr Tyr Pro Gly Thr Ala Asn
    850                 855                 860

Cys Met Asp Ala Glu Gly Lys Leu Arg Pro Asp Gln Asp Tyr Leu Met
865                 870                 875                 880

His Ile Ser His Arg Trp Ser His Asp Gly Ala Arg Gln Thr Ile Ala
                885                 890                 895

Lys Leu Met Gly Ala Gln Asp Gly Ser Gly Asp Ala Val Glu Gln Asp
            900                 905                 910

Val Ala Ser Ser Asn Ala His Cys Val Ala Phe Leu Ile Lys Asp Pro
        915                 920                 925

Gln Lys Val Lys Thr Val Asp Glu Met Arg Glu Arg Leu Arg Met Arg
    930                 935                 940
```

```
Gly Leu Arg Cys His Ile Met Tyr Cys Arg Asn Ser Thr Arg Leu Gln
945                 950                 955                 960

Val Val Pro Leu Leu Ala Ser Arg Ser Gln Ala Leu Arg Tyr Leu Ser
                965                 970                 975

Val Arg Trp Gly Val Ser Val Gly Asn Met Tyr Leu Ile Thr Gly Glu
            980                 985                 990

His Gly Asp Thr Asp Leu Glu Glu Met Leu Ser Gly Leu His Lys Thr
        995                 1000                1005

Val Ile Val Arg Gly Val Thr Glu Lys Gly Ser Glu Ala Leu Val Arg
    1010                1015                1020

Ser Pro Gly Ser Tyr Lys Arg Asp Asp Val Val Pro Ser Glu Thr Pro
1025                1030                1035                1040

Leu Ala Ala Tyr Thr Thr Gly Glu Leu Lys Ala Asp Glu Ile Met Arg
                1045                1050                1055

Ala Leu Lys Gln Val Ser Lys Thr Ser Ser Gly Met
            1060                1065
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Possible peptide encoding sequences"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
WSNATGCCNC CNATHTGGGC NGARGTNATG MGN                              33
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Possible peptide encoding sequences"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

YTNMGNCCNG AYCARGAYTA YYTNATGCAY ATHWSNCAYM GN 42

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide mixture"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGCCNCCNA THTGGGCNGA 20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide mixture"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGCATNAGRT ARTCYTGRTC 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide mixture"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCNGCCCADA TNGGNGGCAT 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide mixture"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAYCARGAYT AYCTNATGCA 20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide mixture"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGRTCNGGNC KNAR 14

What is claimed is:

1. A method of modifying sweetness of a plant part, said method comprising:

growing a plant having integrated into its genome at least one copy of an exogenous DNA sequence encoding a polypeptide having sucrose phosphate synthase activity under conditions whereby said DNA sequence is expressed and sweetness of said plant part is modified.

2. The method of Claim 1, wherein said plant part has an increased level of sucrose as compared to that of a control plant part..

3. The method of Claim 1, wherein said plant part has an increased level of fructose as compared to that of a control plant part.

4. The method of Claim 1, wherein said plant part has an increased level of glucose as compared to that of a control plant part.

5. The method of Claim 1, wherein expression of said DNA sequence is controlled by a tissue specific transcription initiation region.

6. The method of Claim 5, wherein said tissue specific transcription initiation region comprises a fruit specific promoter.

7. The method of Claim 1, wherein said plant is of the family Solenciae.

8. A method of modifying the ratio of soluble solids in a plant sink tissue, said method comprising:

growing a transgenic plant having acid invertase in cells of said sink tissue, said transgenic plant having integrated into its genome at least one copy of an exogeneous DNA sequence encoding a polypeptide having sucrose phosphate synthase activity under conditions whereby said DNA sequence is expressed and the ratio of soluble solids of said plant sink tissue is modified as compared to a control plant sink tissue.

9. The method according to Claim 8, wherein said invertase is endogenous to said cells.

10. A plant sink tissue comprising:

a modified ratio of soluble solids as compared to a control sink tissue, wherein said ratio is modified according to the method of Claim 8.

FIG.1

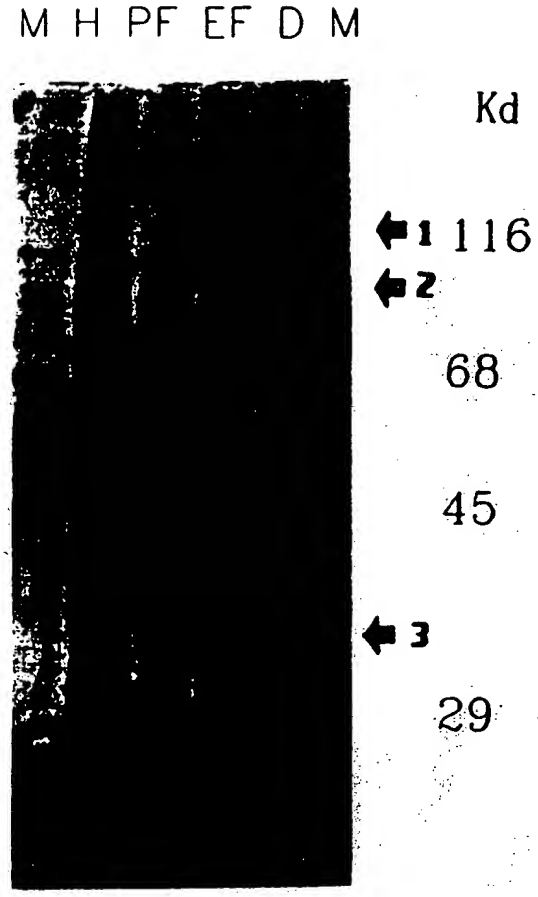


FIG.2

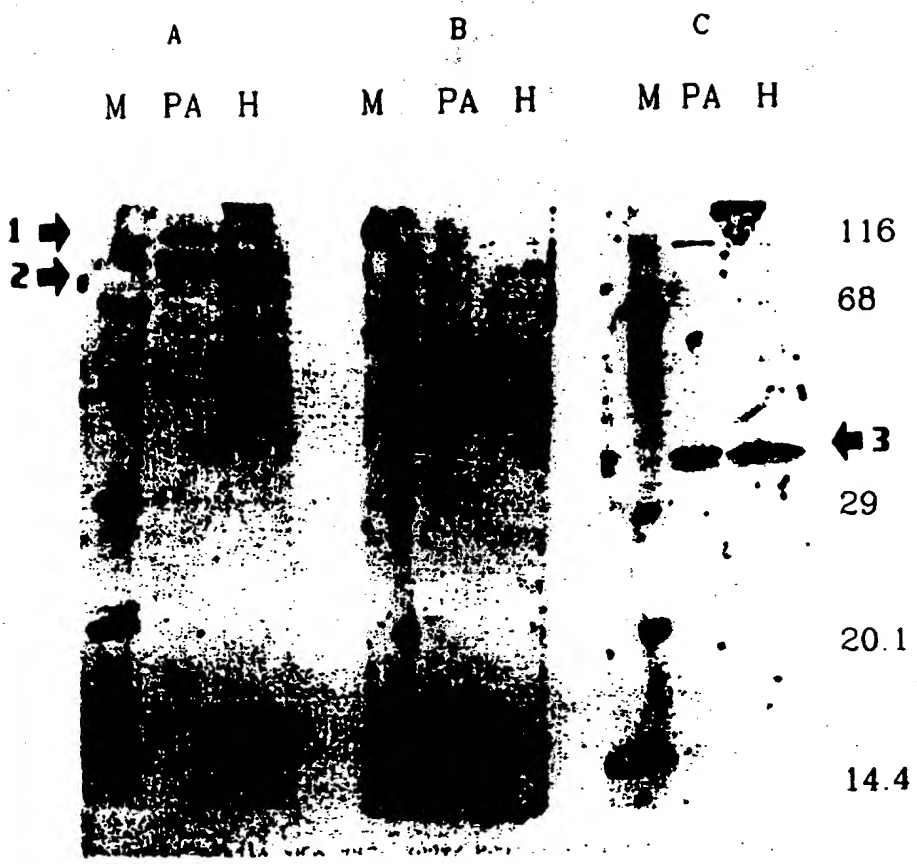


FIG. 3

SPS 90 peptides

A8	ThrTrpIleLys
B4	TyrValValGluLeuAlaArg
B11	SerMetProProIleTrpAlaGluValMetArg

SPS 30 kd peptides

4K	LeuArgProAspGlnAspTyrLeuMetHisIleSerHisArg
12N	TrpSerHisAspGlyAlaArg

FIG. 4

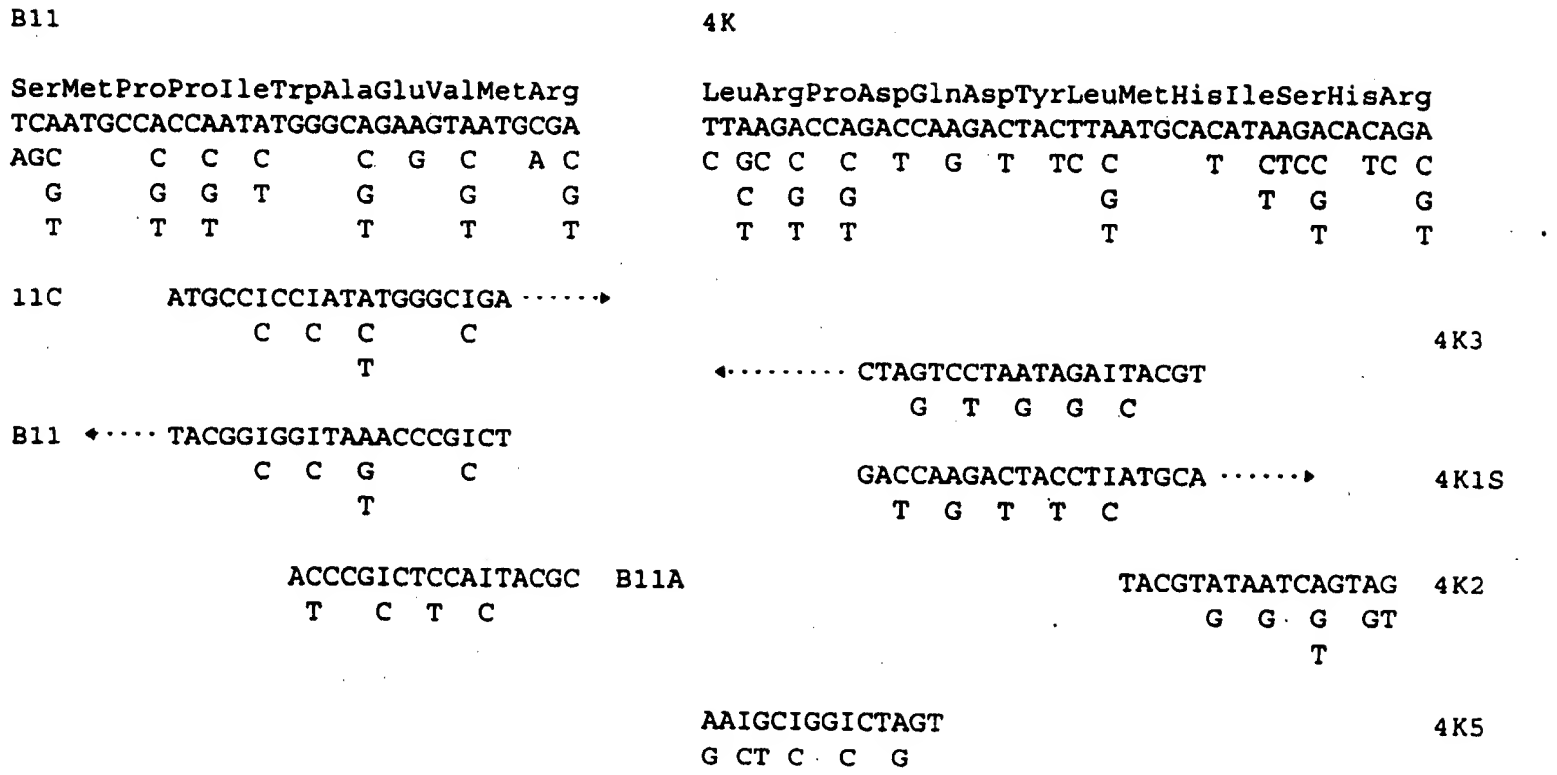


FIG.5A

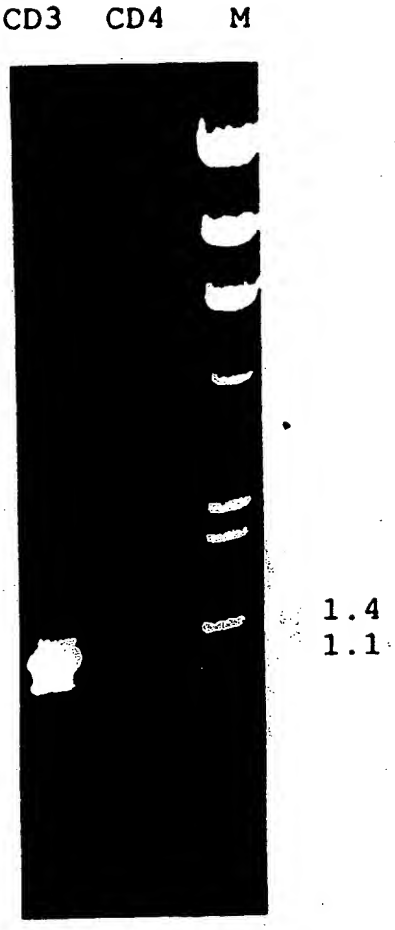


FIG.5B

7/27

FIG.6

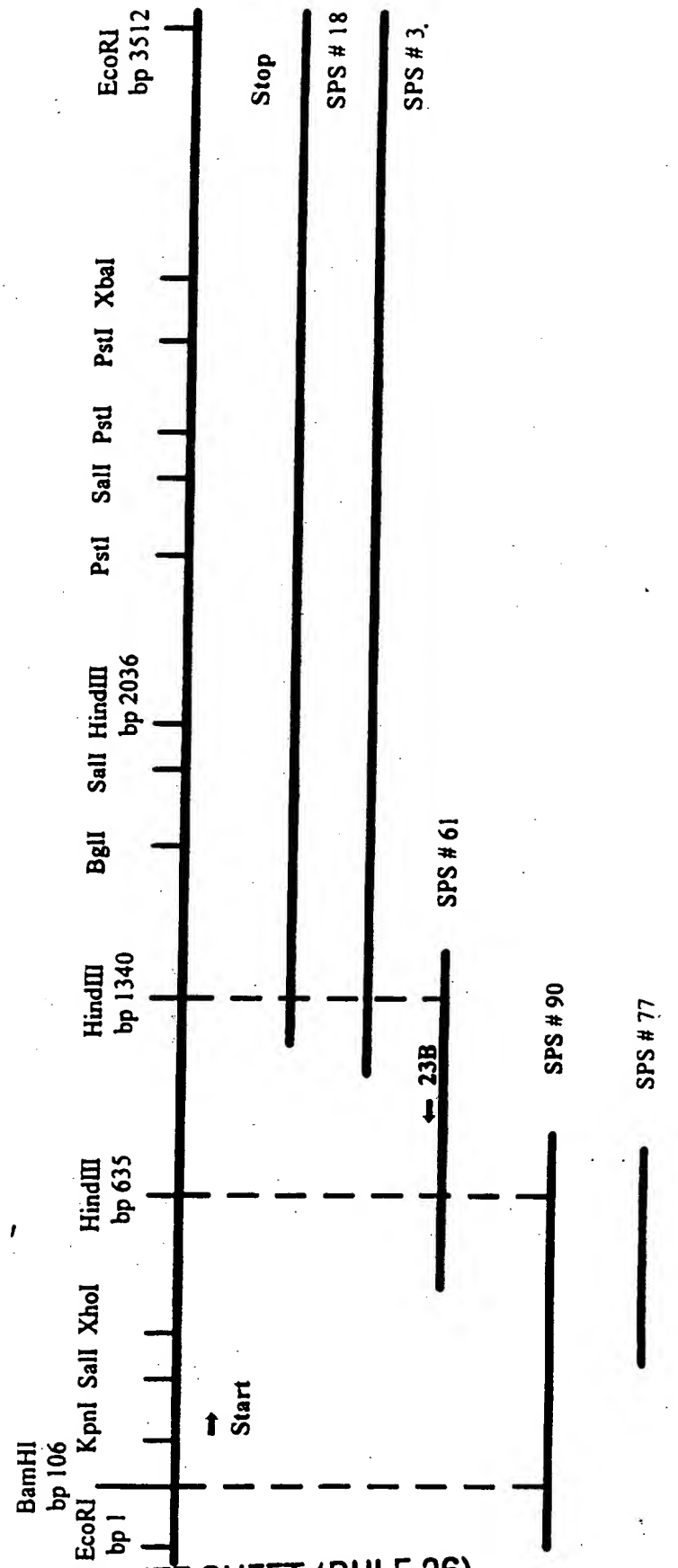

EcoRI bp 1
BamHI bp 106
KpnI
SalI
XhoI
HindIII bp 635
HindIII bp 1340
BglII
SalI
HindIII bp 2036
PstI
SalI
PstI
PstI
XbaI
EcoRI bp 3512
Start
Stop
SPS # 18
SPS # 3
SPS # 61
23B
SPS # 90
SPS # 77

FIG. 7A

```
 EcoRI
 |
GAATTCCGGC GTGGGCGCTG GCTAGTGCT CCCGCAGCGA GCGATCTGAG AGAACGGTAG      60
 2
                                                    BamHI
                                                    |
AGTTCCGGCC GGGCGCGCGG GAGAGGAGGA GGGTCGGGCG GGGAGGATCC G ATG GCC      117
                                                    106     MET Ala
                                  KpnI
                                  |
GGG AAC GAG TGG ATC AAT GGG TAC CTG GAG GCG ATC CTC GAC AGC CAC       165
Gly Asn Glu Trp Ile Asn Gly Tyr Leu Glu Ala Ile Leu Asp Ser His
                                  142

ACC TCG TCG CGG GGT GCC GGC GGC GGC GGC GGC GGG GGG GAC CCC AGG       213
Thr Ser Ser Arg Gly Ala Gly Gly Gly Gly Gly Gly Gly Asp Pro Arg

TCG CCG ACG AAG GCG GCG AGC CCC CGC GGC GCG CAC ATG AAC TTC AAC       261
Ser Pro Thr Lys Ala Ala Ser Pro Arg Gly Ala His Met Asn Phe Asn

                                                    SalI
                                                    |
CCC TCG CAC TAC TTC GTC GAG GAG GTG GTC AAG GGC GTC GAC GAG AGC       309
Pro Ser His Tyr Phe Val Glu Glu Val Val Lys Gly Val Asp Glu Ser
                                                    299

GAC CTC CAC CGG ACG TGG ATC AAG GTC GTC GCC ACC CGC AAC GCC CGC       357
Asp Leu His Arg Thr Trp Ile Lys Val Val Ala Thr Arg Asn Ala Arg
                 AB
```


WO 97/15678 PCT/US96/17351

8/27

FIG. 7B

```
                          XhoI
                           |
GAG CGC AGC ACC AGG CTC GAG AAC ATG TGC TGG CGG ATC TGG CAC CTC      405
Glu Arg Ser Thr Arg Leu Glu Asn Met Cys Trp Arg Ile Trp His Leu
                          374

GCG CGC AAG AAG AAG CAG CTG GAG CTG GAG GGC ATC CAG AGA ATC TCG      453
Ala Arg Lys Lys Lys Gln Leu Glu Leu Glu Gly Ile Gln Arg Ile Ser

GCA AGA AGG AAG GAA CAG GAG CAG GTG CGT CGT GAG GCG ACG GAG GAC      501
Ala Arg Arg Lys Glu Gln Glu Gln Val Arg Arg Glu Ala Thr Glu Asp

CTG GCC GAG GAT CTG TCA GAA GGC GAG AAG GGA GAC ACC ATC GGC GAG      549
Leu Ala Glu Asp Leu Ser Glu Gly Glu Lys Gly Asp Thr Ile Gly Glu

CTT GCG CCG GTT GAG ACG ACC AAG AAG AAG TTC CAG AGG AAC TTC TCT      597
Leu Ala Pro Val Glu Thr Thr Lys Lys Lys Phe Gln Arg Asn Phe Ser

                                                  HindIII
                                                    |
GAC CTT ACC GTC TGG TCT GAC GAC AAT AAG GAG AAG AAG CTT TAC ATT      645
Asp Leu Thr Val Trp Ser Asp Asp Asn Lys Glu Lys Lys Leu Tyr Ile
                                                 635

GTG CTC ATC AGC GTG CAT GGT CTT GTT CGT GGA GAA AAC ATG GAA CTA      693
Val Leu Ile Ser Val His Gly Leu Val Arg Gly Glu Asn Met Glu Leu
```


WO 97/15678

9/27

PCT/US96/17351

FIG. 7C

```
GGT CGT GAT TCT GAT ACA GGT GGC CAG GTG AAA TAT GTG GTC GAA CTT      741
Gly Arg Asp Ser Asp Thr Gly Gly Gln Val Lys Tyr Val Val Glu Leu
                                                    B4

GCA AGA GCG ATG TCA ATG ATG CCT GGA GTG TAC AGG GTG GAC CTC TTC      789
Ala Arg Ala Met Ser Met Met Pro Gly Val Tyr Arg Val Asp Leu Phe

ACT CGT CAA GTG TCA TCT CCT GAC GTG GAC TGG AGC TAC GGT GAG CCA      837
Thr Arg Gln Val Ser Ser Pro Asp Val Asp Trp Ser Tyr Gly Glu Pro

ACC GAG ATG TTA TGC GCC GGT TCC AAT GAT GGA GAG GGG ATG GGT GAG      885
Thr Glu Met Leu Cys Ala Gly Ser Asn Asp Gly Glu Gly Met Gly Glu

AGT GGC GGA GCC TAC ATT GTG CGC ATA CCG TGT GGG CCG CGG GAT AAA      933
Ser Gly Gly Ala Tyr Ile Val Arg Ile Pro Cys Gly Pro Arg Asp Lys

TAC CTC AAG AAG GAA GCG TTG TGG CCT TAC CTC CAA GAG TTT GTC GAT      981
Tyr Leu Lys Lys Glu Ala Leu Trp Pro Tyr Leu Gln Glu Phe Val Asp

GGA GCC CTT GCG CAT ATC CTG AAC ATG TCC AAG GCT CTG GGA GAG CAG     1029
Gly Ala Leu Ala His Ile Leu Asn Met Ser Lys Ala Leu Gly Glu Gln

GTT GGA AAT GGG AGG CCA GTA CTG CCT TAC GTG ATA CAT GGG CAC TAT     1077
Val Gly Asn Gly Arg Pro Val Leu Pro Tyr Val Ile His Gly His Tyr

GCC GAT GCT GGA GAT GTT GCT GCT CTC CTT TCT GGT GCG CTG AAT GTG     1125
Ala Asp Ala Gly Asp Val Ala Ala Leu Leu Ser Gly Ala Leu Asn Val
```

WO 97/15678

10/27

PCT/US96/17351

FIG. 7D

```
CCA ATG GTG CTC ACT GGC CAC TCA CTT GGG AGG AAC AAG CTG GAA CAA      1173
Pro Met Val Leu Thr Gly His Ser Leu Gly Arg Asn Lys Leu Glu Gln

CTG CTG AAG CAA GGG CGC ATG TCC AAG GAG GAG ATC GAT TCG ACA TAC      1221
Leu Leu Lys Gln Gly Arg Met Ser Lys Glu Glu Ile Asp Ser Thr Tyr

AAG ATC ATG AGG CGT ATC GAG GGT GAG GAG CTG GCC CTG GAT GCG TCA      1269
Lys Ile Met Arg Arg Ile Glu Gly Glu Glu Leu Ala Leu Asp Ala Ser

GAG CTT GTA ATC ACG AGC ACA AGG CAG GAG ATT GAT GAG CAG TGG GGA      1317
Glu Leu Val Ile Thr Ser Thr Arg Gln Glu Ile Asp Glu Gln Trp Gly

                          HindIII
                          |
TTG TAC GAT GGA TTT GAT GTC AAG CTT GAG AAA GTG CTG AGG GCA CGG      1365
Leu Tyr Asp Gly Phe Asp Val Lys Leu Glu Lys Val Leu Arg Ala Arg
                          1340

                          NcoI
                          |
GCG AGG CGC GGG GTT AGC TGC CAT GGT CGT TAC ATG CCT AGG ATG GTG      1413
Ala Arg Arg Gly Val Ser Cys His Gly Arg Tyr Met Pro Arg Met Val
                          1387

GTG ATT CCT CCG GGA ATG GAT TTC AGC AAT GTT GTA GTT CAT GAA GAC      1461
Val Ile Pro Pro Gly Met Asp Phe Ser Asn Val Val Val His Glu Asp
```


WO 97/15678

11/27

PCT/US96/17351

FIG. 7E

```
ATT GAT GGG GAT GGT GAC GTC AAA GAT GAT ATC GTT GGT TTG GAG GGT      1509
Ile Asp Gly Asp Gly Asp Val Lys Asp Asp Ile Val Gly Leu Glu Gly

GCC TCA CCC AAG TCA ATG CCC CCA ATT TGG GCC GAA GTG ATG CGG TTC      1557
Ala Ser Pro Lys Ser Met Pro Pro Ile Trp Ala Glu Val Met Arg Phe
                                  B11

CTG ACC AAC CCT CAC AAG CCG ATG ATC CTG GCG TTA TCA AGA CCA GAC      1605
Leu Thr Asn Pro His Lys Pro Met Ile Leu Ala Leu Ser Arg Pro Asp

CCG AAG AAG AAC ATC ACT ACC CTC GTC AAA GCC TTT GGA GAG TGT CGT      1653
Pro Lys Lys Asn Ile Thr Thr Leu Val Lys Ala Phe Gly Glu Cys Arg

CCA CTC AGG GAA CTT GCA AAC CTT ACT CTG ATC ATG GGT AAC AGA GAT      1701
Pro Leu Arg Glu Leu Ala Asn Leu Thr Leu Ile Met Gly Asn Arg Asp

GAC ATC GAC GAC ATG TCT GCT GGC AAT GCC AGT GTC CTC ACC ACA GTT      1749
Asp Ile Asp Asp Met Ser Ala Gly Asn Ala Ser Val Leu Thr Thr Val

CTG AAG CTG ATT GAC AAG TAT GAT CTG TAC GGA AGC GTG GCG TTC CCT      1797
Leu Lys Leu Ile Asp Lys Tyr Asp Leu Tyr Gly Ser Val Ala Phe Pro

                                  Bg1II
                                  |
AAG CAT CAC AAT CAG GCT GAC GTC CCG GAG ATC TAT CGC CTC GCG GCC      1845
Lys His His Asn Gln Ala Asp Val Pro Glu Ile Tyr Arg Leu Ala Ala
                                  1827
```


12/27

WO 97/15678

PCT/US96/17351

FIG. 7F

```
AAA ATG AAG GGC GTC TTC ATC AAC CCT GCT CTC GTT GAG CCG TTT GGT        1893
Lys Met Lys Gly Val Phe Ile Asn Pro Ala Leu Val Glu Pro Phe Gly

CTC ACC CTG ATC GAG GCT GCG GCA CAC GGA CTC CCG ATA GTC GCT ACC        1941
Leu Thr Leu Ile Glu Ala Ala Ala His Gly Leu Pro Ile Val Ala Thr

                    SalI
                     |
AAG AAT GGT GGT CCG GTC GAC ATT ACA AAT GCA TTA AAC AAC GGA CTG        1989
Lys Asn Gly Gly Pro Val Asp Ile Thr Asn Ala Leu Asn Asn Gly Leu
                    1958

                                                        HindIII
                                                           |
CTC GTT GAC CCA CAC GAC CAG AAC GCC ATC GCT GAT GCA CTG CTG AAG        2037
Leu Val Asp Pro His Asp Gln Asn Ala Ile Ala Asp Ala Leu Leu Lys
                                                          2036

CTT GTG GCA GAC AAG AAC CTG TGG CAG GAA TGC CGG AGA AAC GGG CTG        2085
Leu Val Ala Asp Lys Asn Leu Trp Gln Glu Cys Arg Arg Asn Gly Leu

CGC AAC ATC CAC CTC TAC TCA TGG CCG GAG CAC TGC CGC ACT TAC CTC        2133
Arg Asn Ile His Leu Tyr Ser Trp Pro Glu His Cys Arg Thr Tyr Leu

ACC AGG GTG GCC GGG TGC CGG TTA AGG AAC CCG AGG TGG CTG AAG GAC        2181
Thr Arg Val Ala Gly Cys Arg Leu Arg Asn Pro Arg Trp Leu Lys Asp
```


WO 97/15678

13/27

PCT/US96/17351

FIG. 7G

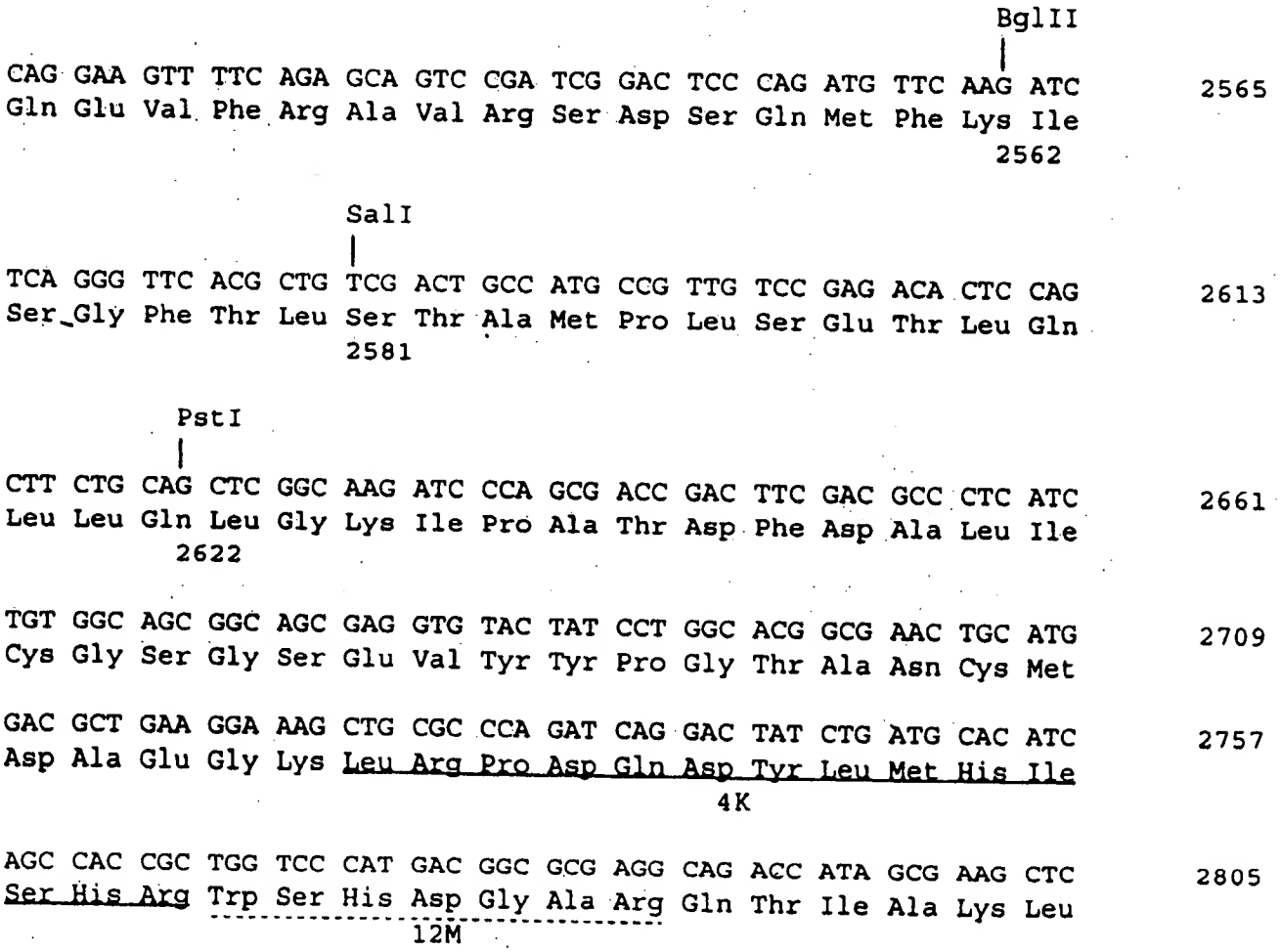
```
                                                                NcoI
                                                                 |
ACA CCA GCA GAT GCC GGA GCC GAT GAG GAG GAG TTC CTG GAG GAT TCC      2229
Thr Pro Ala Asp Ala Gly Ala Asp Glu Glu Glu Phe Leu Glu Asp Ser
                                                              2229

ATG GAC GCT CAG GAC CTG TCA CTC CGT CTG TCC ATC GAC GGT GAG AAG      2277
Met Asp Ala Gln Asp Leu Ser Leu Arg Leu Ser Ile Asp Gly Glu Lys

AGC TCG CTG AAC ACT AAC GAT CCA CTG TGG TTC GAC CCC CAG GAT CAA      2325
Ser Ser Leu Asn Thr Asn Asp Pro Leu Trp Phe Asp Pro Gln Asp Gln

GTG CAG AAG ATC ATG AAC AAC ATC AAG CAG TCG TCA GCG CTT CCT CCG      2373
Val Gln Lys Ile Met Asn Asn Ile Lys Gln Ser Ser Ala Leu Pro Pro

TCC ATG TCC TCA GTC GCA GCC GAG GGC ACA GGC AGC ACC ATG AAC AAA      2421
Ser Met Ser Ser Val Ala Ala Glu Gly Thr Gly Ser Thr Met Asn Lys

TAC CCA CTC CTG CGC CGG CGC CGG CGC TTG TTC GTC ATA GCT GTG GAC      2469
Tyr Pro Leu Leu Arg Arg Arg Arg Arg Leu Phe Val Ile Ala Val Asp

                                                        PstI
                                                         |
TGC TAC CAG GAC GAT GGC CGT GCT AGC AAG AAG ATG CTG CAG GTG ATC      2517
Cys Tyr Gln Asp Asp Gly Arg Ala Ser Lys Lys Met Leu Gln Val Ile
                                                      2511
```


WO 97/15678

14/27

PCT/US96/17351

FIG. 7H



15/27

WO 97/15678
PCT/US96/17351

FIG. 7I

```
ATG GGC GCT CAG GAC GGT TCA GGC GAC GCT GTC GAG CAG GAC GTG GCG      2853
Met Gly Ala Gln Asp Gly Ser Gly Asp Ala Val Glu Gln Asp Val Ala

TCC AGT AAT GCA CAC TGT GTC GCG TTC CTC ATC AAA GAC CCC CAA AAG      2901
Ser Ser Asn Ala His Cys Val Ala Phe Leu Ile Lys Asp Pro Gln Lys

GTG AAA ACG GTC GAT GAG ATG AGG GAG CGG CTG AGG ATG CGT GGT CTC      2949
Val Lys Thr Val Asp Glu Met Arg Glu Arg Leu Arg Met Arg Gly Leu

                                PstI
                                 |
CGC TGC CAC ATC ATG TAC TGC AGG AAC TCG ACA AGG CTT CAG GTT GTC      2997
Arg Cys His Ile Met Tyr Cys Arg Asn Ser Thr Arg Leu Gln Val Val
                                2972

CCT CTG CTA GCA TCA AGG TCA CAG GCA CTC AGG TAT CTT TCC GTG CGC      3045
Pro Leu Leu Ala Ser Arg Ser Gln Ala Leu Arg Tyr Leu Ser Val Arg

TGG GGC GTA TCT GTG GGG AAC ATG TAT CTG ATC ACC GGG GAA CAT GGC      3093
Trp Gly Val Ser Val Gly Asn Met Tyr Leu Ile Thr Gly Glu His Gly

            XbaI
             |
GAC ACC GAT CTA GAG GAG ATG CTA TCC GGG CTA CAC AAG ACC GTG ATC      3141
Asp Thr Asp Leu Glu Glu Met Leu Ser Gly Leu His Lys Thr Val Ile
            3103
```


16/27

FIG. 7J

```
GTC CGT GGC GTC ACC GAG AAG GGT TCG GAA GCA CTG GTG AGG AGC CCA      3189
Val Arg Gly Val Thr Glu Lys Gly Ser Glu Ala Leu Val Arg Ser Pro

GGA AGC TAC AAG AGG GAC GAT GTC GTC CCG TCT GAG ACC CCC TTG GCT      3237
Gly Ser Tyr Lys Arg Asp Asp Val Val Pro Ser Glu Thr Pro Leu Ala

GCG TAC ACG ACT GGT GAG CTG AAG GCC GAC GAG ATC ATG CGG GCT CTG      3285
Ala Tyr Thr Thr Gly Glu Leu Lys Ala Asp Glu Ile Met Arg Ala Leu

AAG CAA GTC TCC AAG ACT TCC AGC GGC ATG TGAATTTGAT GCTTCTTTTA        3335
Lys Gln Val Ser Lys Thr Ser Ser Gly Met

CATTTTGTCCTTTTCTTCACTGCTATATAAAATAAGTTGTGAACAGTACCGCGGGTGTGT        3395

ATATATATATTGCAGTGACAAATAAAACAGGACACTGCTAACTATACTGGTGAATATACG        3455

                                                  EcoRI
                                                    |
ACTGTCAAGATTGTATGCTAAGTACTCCATTTCTCAATGTATCAATCGGAATTC              3509
                                                  3505
```


WO 97/15678

17/27

PCT/US96/17351

FIG.8

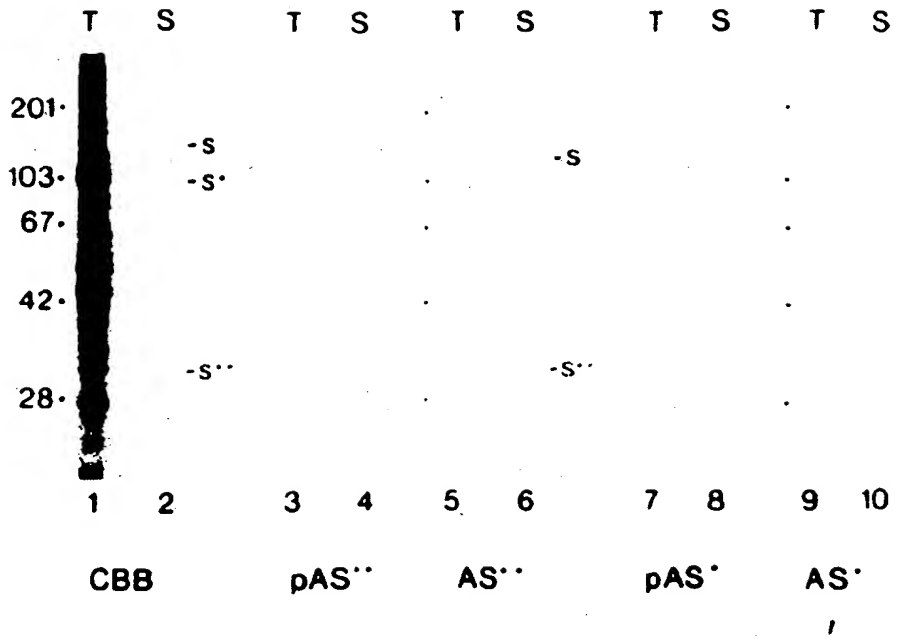

T S T S T S T S T S
201
103
67
42
28
-S
-S
-S
-S
-S
1 2 3 4 5 6 7 8 9 10
CBB
pAS
AS
pAS
AS

FIG.9A

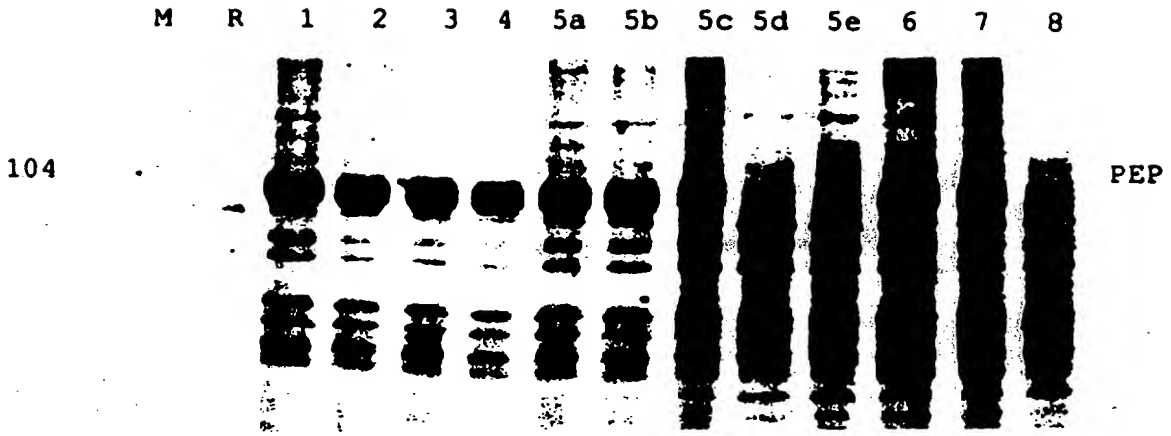


FIG.9B

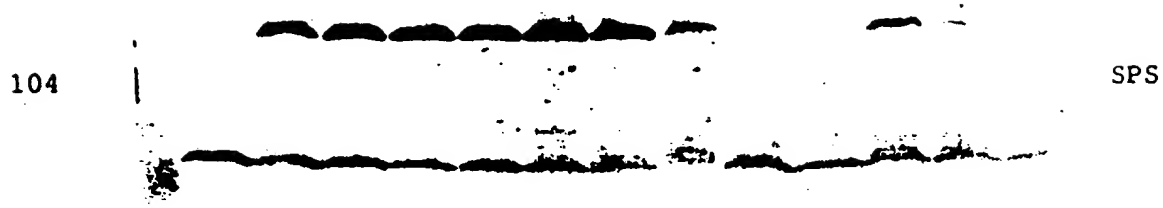


FIG. 10A

PCGN627

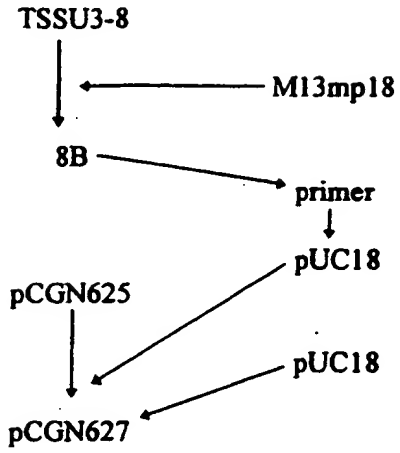


FIG. 10B

pCGN639

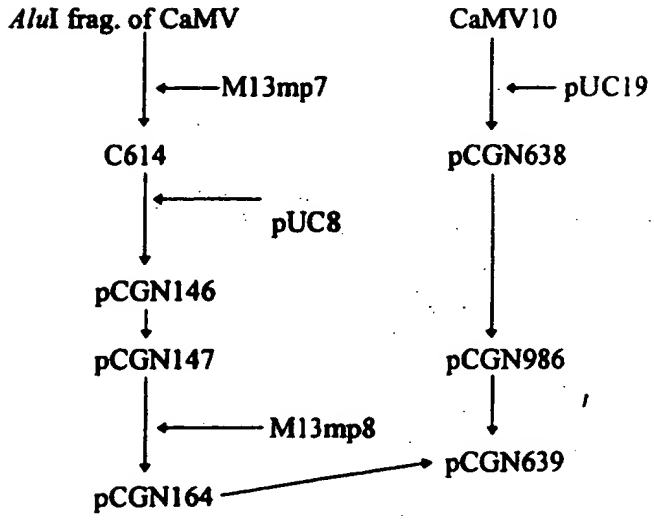
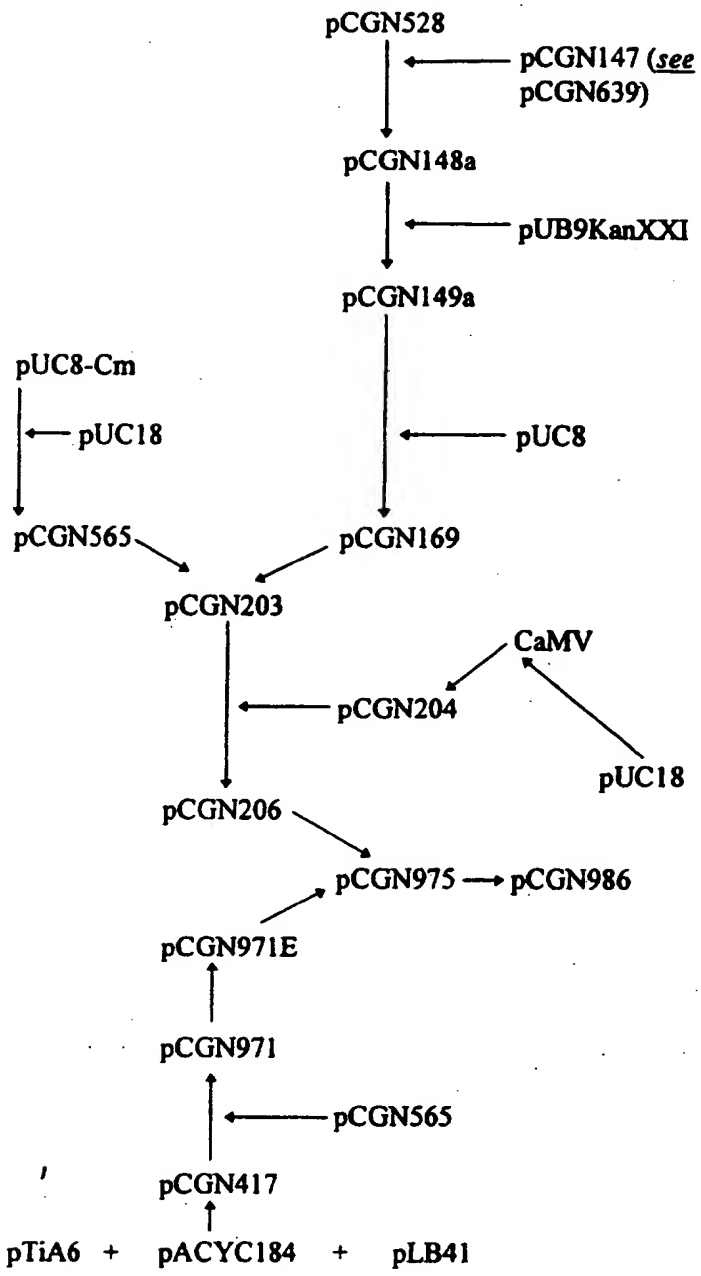


FIG. 10C

pCGN986



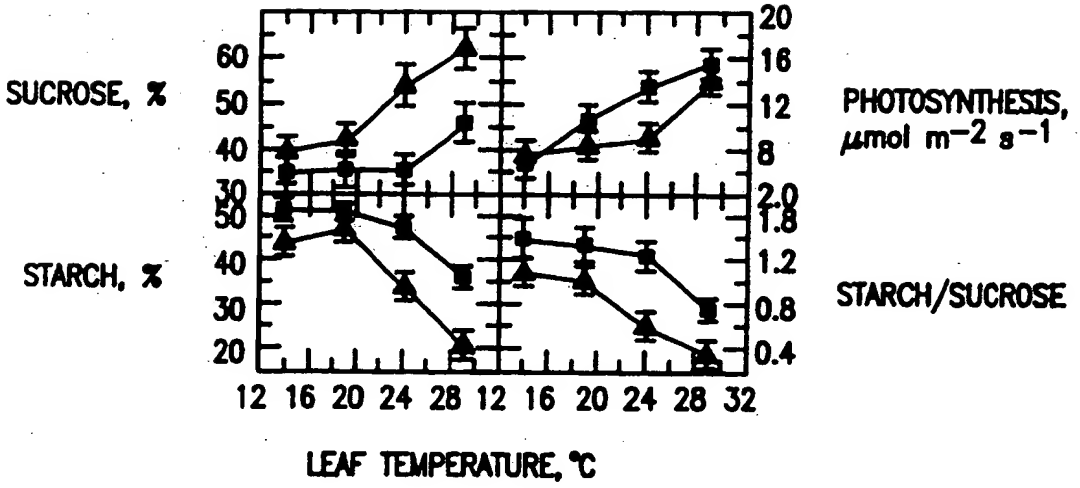

SUCROSE, %
STARCH, %
PHOTOSYNTHESIS, μmol m⁻² s⁻¹
STARCH/SUCROSE
LEAF TEMPERATURE, °C
60
50
40
30
20
16
12
8
2.0
1.8
1.2
0.8
0.4
12 16 20 24 28 12 16 20 24 28 32

FIG. 11

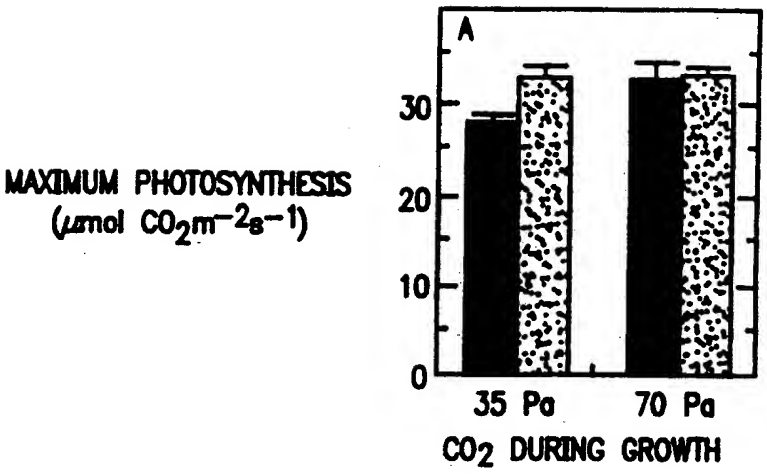

MAXIMUM PHOTOSYNTHESIS
(μmol $CO_2 m^{-2} s^{-1}$)
A
30
20
10
0
35 Pa
70 Pa
CO_2 DURING GROWTH

FIG. 12A

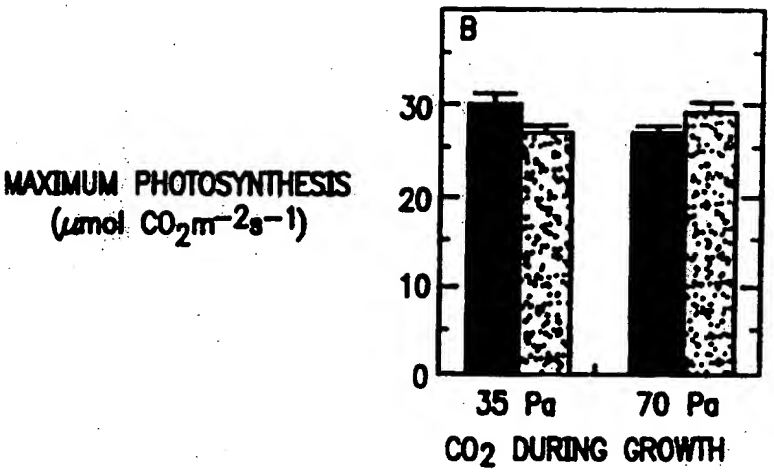

B
MAXIMUM PHOTOSYNTHESIS
(μmol $CO_2 m^{-2} s^{-1}$)
30
20
10
0
35 Pa
70 Pa
CO_2 DURING GROWTH

FIG. 12B

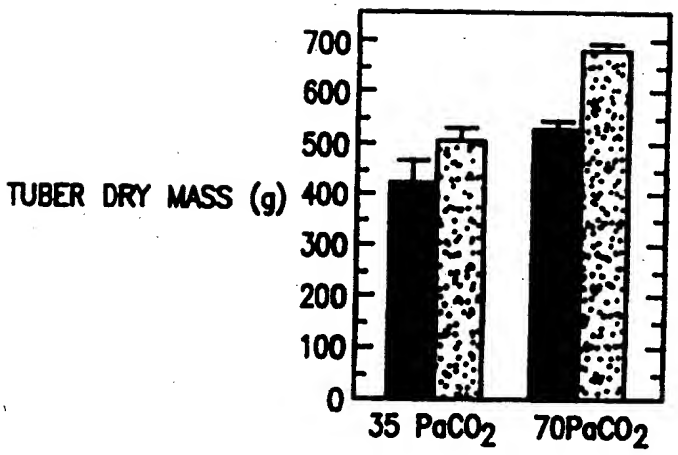


FIG. 13A

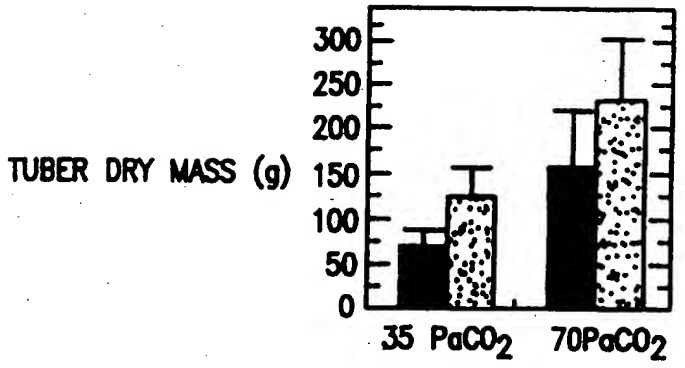


FIG. 13B

BLANK PAGE

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☑ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☑ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

BLANK PAGE